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THE COMPARATIVE ANTIRACHITIC EFFICIENCY OF IRRADIATED ERGOSTEROL, IRRADIATED YEAST, AND COD LIVER OIL FOR THE CHICKEN*

By

R. M. BETHKE, P. R. RECORD,† AND D. C. KENNARD

(From the Department of Animal Industry, Ohio Agricultural Experiment Station, Wooster)

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THE effectiveness of cod liver oil and ultraviolet rays as prophylactic agents against rickets in the chicken has been definitely established. That irradiated preparations such as irradiated ergosterol and yeast are not so effective in the control of rickets in the chick as is cod liver oil when equivalent rat units of vitamin D are fed has also been reported by several investigators.

Mussehl and Ackerson (1) in 1930 reported that amounts of irradiated yeast and irradiated ergosterol theoretically equivalent to 10 and 50 per cent of cod liver oil, respectively, did not prevent leg weakness. At about the same time Massengale and Mussmeier (2) found that it was necessary to administer the equivalent of 200 per cent cod liver oil in the form of activated ergosterol to produce the effects of 2 per cent of cod liver oil. Hess and Supplee (3) and Russell and Klein (4) observed that it required more units of vitamin D in the form of irradiated ergosterol than in the form of cod liver oil to produce normal calcification in the chick.

Recently Steenbock, Kletzien, and Halpin (5) reported that it required from 40 to 120 per cent cod liver oil equivalence as irradiated ergosterol and from 7.5 to 60 per cent cod liver oil equivalence as irradiated yeast to produce the same degree of bone formation as 1 per cent of cod liver oil of average potency. The same investigators found no difference in the antirachitic efficiency or the degree of toxicity of irradiated ergosterol fed

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† Acetol Products Fellow. This investigation was conducted under a fellowship grant from Acetol Products, Inc., New York City.

in corn oil or in cod liver oil and accordingly concluded that vitamin D produced by ordinary irradiation of ergosterol with a quartz mercury vapor lamp is a different substance from that found in cod liver oil.

In our series of experiments, started in the fall of 1928, we attempted to compare the effectiveness of vitamin D in the form of irradiated ergosterol and irradiated yeast with ultraviolet light and cod liver oil and to determine, if possible, the reason for the difference in efficiency of the irradiated sterol and the natural vitamin D of cod liver oil.

Experimental

Early in the investigation it was realized that irradiated ergosterol dissolved in linseed oil was not as effective in the treatment of rickets in chicks as in rats. It was also realized that in order to obtain an absolute comparison between the efficiency of cod liver oil and irradiated ergosterol and yeast the products would have to be fed on a known unit basis. Cod liver oil may vary as much as 100 per cent in vitamin D potency, whereas the activity of irradiated ergosterol or yeast may vary several thousand per cent. Accordingly, it is fundamental that the potency of the products be known and that the comparisons be made on a minimum unit protective basis if we are to draw intelligent conclusions.

In all our work the vitamin D potency of the cod liver oils, irradiated yeast, and solutions of irradiated ergosterol were determined on rats in our laboratory according to the Wisconsin Alumni Research Foundation procedure, and the activity expressed in Steenbock units.

The first experiments were of the prophylactic and curative type. The prophylactic series consisted of 5 lots, of White Leghorn chicks each, started on their respective rations when day-old. In the curative series, chicks which showed distinct symptoms of leg weakness at 7 weeks, while on the control ration, were divided into 5 lots of 18 birds each. All lots received a ration of ground yellow corn 52, ground wheat 20, wheat bran 5, dried buttermilk 20, calcium carbonate 2, and sodium chloride 1. This ration analyzed 1.02 per cent calcium and 0.45 per cent phosphorus. Varying amounts of irradiated ergosterol dissolved in linseed oil were incorporated in the rations of the lots in the prophylactic series, whereas in the curative trials definite amounts of the sterol in oil were fed in gelatin capsules daily. A negative control lot and a lot exposed to ultra violet light were included in both series.

The chicks in the prophylactic series were on experiment for 8 weeks, when 10 representative birds from each lot were killed for blood and bone

ash analysis. In the curative series, one-half of the chicks in each lot were killed after 10 days of supplemental feeding and the remainder at 19 days. The results of both series are recorded in Table I.

It required between 40 and 250 rat units of vitamin D per 100 gm. of basal ration as irradiated ergosterol to prevent leg weakness. On the other

TABLE I

THE ANTIRACHITIC EFFICIENCY OF IRRADIATED ERGOSTEROL IN LINSEED OIL FOR CHICKS

Prophylactic Trial

Ration supplements	Vitamin D rat units per 100 gm. ration	Average final weight	Blood analysis		Average ash in tibia
			Ca. per 100 cc. serum	P. per 100 cc. serum	
		gm.	mg.	mg.	per cent
None (control)	0	301	8.7	5.6	42.9
4 cc. linseed-ergosterol solution (1) per kilo	20	348	9.9	6.0	43.8
8 cc. linseed-ergosterol solution (1) per kilo	40	370	10.8	6.6	47.7
10 cc. linseed-ergosterol solution (2) per kilo	250	401	12.2	7.9	52.7
Ultra violet irradiation 10 min. daily	?	395	12.4	8.0	52.7

Curative Trial

(Chicks on basal ration for 7 weeks before change)

Ration supplements	Vitamin D units fed daily	Days after chg.	Av. final wt.	Blood Analysis		Average ash in tibia
				Ca. per 100 cc. serum	P. per 100 cc. serum	
			gm.	mg.	mg.	per cent
		0	310	8.7	5.7	42.2
None	0	10	322	9.5	4.2	42.7
		19	351	8.9	4.8	43.0
0.2 cc. linseed-ergosterol solution (1) per		10	352	9.9	4.5	42.1
chick daily	10	19	427	10.3	4.9	43.5
0.4 cc. linseed-ergosterol solution (1) per		10	347	9.7	4.9	43.3
chick daily	20	19	446	9.5	5.2	45.1
0.4 cc. linseed-ergosterol solution (2) per	100	10	377	9.6	4.8	44.4
chick daily*		19	479	11.9	5.3	48.0
Ultra violet irradiation 10 min. daily	?	10	356	11.3	5.0	44.9
		19	442	11.2	6.6	49.6

* Solution 2 was five times as concentrated as solution 1.

hand, 20 units daily for a period of 19 days was not sufficient for curing rickets, since a few birds were still affected at the termination of the experiment. All the birds in the lot that received 100 vitamin D units daily, or were subjected to ultraviolet irradiation, had completely recovered by the 19th day. These observations were substantiated by the per cent of bone ash.

Both the prophylactic and curative trials reflected the comparative inefficiency of irradiated ergosterol as an anti-rachitic substance for chicks. In seeking for a possible explanation of the results obtained, it occurred to us that the difficulty, in part, might have been due to the sample of ergosterol or the type of basal ration, which, in our estimation, was too low in phosphorus for practical purposes. Accordingly, another series of several experiments, which involved new samples of irradiated ergosterol and a different rachitic ration, were carried out. The basal ration used throughout was one which gave good growth when properly supplemented with vitamin D. It consisted of ground yellow corn 46, ground wheat 20, wheat bran 5, soybean oilmeal 20, dried buttermilk 5, steamed bone meal 3, and sodium chloride 1. This ration in the different experiments varied between 1.12–1.36 per cent calcium and 0.88–0.98 per cent phosphorus, or a Ca:P ratio of 1.27–1.40.

The chicks in all trials were White Leghorns. They were started on their respective rations when day-old. They were of our own hatching and came from parent stock which had regularly been fed 1 per cent of cod liver oil in their ration. In all instances the chicks were brooded indoors on hardware cloth. Special precautions were taken to exclude all sunlight, by covering the windows with heavy brown wrapping paper. At the start of each trial a few more chicks than were to be carried through the experiment were placed in each lot. All lots were culled to an equal number at the end of seven days, by eliminating those chicks which showed any signs of weakness.

At the end of six weeks, ten or more representative chicks, both males and females, were selected from each lot and killed for blood and bone analysis. The blood from each lot was pooled, refrigerated over night, and the serum analyzed for calcium (6) and inorganic phosphorus (7). Both tibiae from each bird were saved for ash determinations. In removing the adhering flesh, the proximal cartilage was also removed. The bones were then dried, crushed, and wrapped in individual filter papers and extracted in Soxhlet extractors with alcohol and ether. The per cent ash is expressed on a moisture-and fat-free basis.

The first experiment in this series was in part a repetition of the initial work with the inclusion of a cod-liver-oil-fed lot. It consisted of 6 lots of 25 chicks each. A new solution of irradiated ergosterol in linseed oil was used. The ergosterol solutions were diluted so that 1 per cent of the oil solution furnished the respective number of rat units. The results are shown in Table II.

TABLE II

THE EFFECTIVENESS OF IRRADIATED ERGOSTEROL FOR CALCIFICATION IN THE CHICK

Additions to basal ration	Vitamin D rat units per 100 gm. ration	Average final weight	Blood analysis		Average ash in tibia
			Ca. per 100 cc. serum	P. per 100 cc. serum	
		gm.	mg.	mg.	per cent
None (negative control)	0	174	7.1	7.1	42.3
Ultra violet irradiation*	?	286	9.2	7.7	51.8
2.00 per cent cod liver oil	50	288	10.0	7.8	51.1
0.25 per cent ergosterol-linseed oil solution	83	268	9.2	8.1	49.7
0.50 per cent ergosterol-linseed oil solution	166	299	10.8	8.2	50.8
1.00 per cent ergosterol-linseed oil solution	332	312	9.3	8.0	51.5

* Chicks irradiated for 10 minutes three times per week.

The chicks in the negative control lot gave evidence of leg weakness at the fourth week and at six weeks all the birds were severely affected. No external symptoms of leg weakness were observed in any of the other lots except two cases in the lot that received 83 units of vitamin D as irradiated ergosterol in 100 gm. of ration. These observations are substantiated by the bone ash values. The blood analysis of all lots was normal, except the negative control lot, which showed lowered calcium and phosphorus. The results showed that it required between 83 and 166 rat units of vitamin D as irradiated ergosterol per 100 gm. of feed to protect the chick against rickets.

The results of the two trials, in general, were in accord with the findings of other investigators; that it required more equivalent rat units of vitamin D in the form of irradiated ergosterol than cod liver oil. The data, however, did not lend themselves to a direct comparison of the number of units of the vitamin that were required from cod liver oil or the irradiated sterol; since the cod liver oil (2 per cent) apparently had been fed in excess of requirements. Before any fair comparison of the two products could be

made, it was necessary to determine the minimum number of units of either source of vitamin D that were required for normal calcification. It also occurred to us that cod liver oil might carry some factor which would enhance the antirachitic action of the irradiated ergosterol.

To answer these questions 12 lots of 20 chicks each were started on experiment. Three lots were fed the basal ration supplemented with 0.25, 0.50, and 1.00 per cent of cod liver oil. The latter represented a new sample which had a potency of 28 rat units per gram. To the ration of four other lots were added 0.01, 0.05, 0.10, and 0.25 per cent of the same cod liver oil to which had been added irradiated ergosterol to furnish 2, 10, 20, and 50 rat units of vitamin D per 100 gm. of ration, respectively; in addition to the units supplied by the cod liver oil. Four other lots received the basal ration fortified with such amounts of irradiated ergosterol dissolved in corn oil to furnish 1.5, 36, 72, and 143 rat units per 100 gm. of ration, respectively. The remaining lot served as a no-vitamin D control. All three products were evaporated on the basal ration with ether. Fresh feed was prepared every 7–10 days. The results are recorded in Table III.

The chicks in Lots 1, 5, 9, and 10, which received the unsupplemented ration, 2 units of irradiated ergosterol in cod liver oil, and 1.5 and 36 units of activated sterol in corn oil per 100 gm. of ration, respectively, showed marked symptoms of rickets, which were reflected in the low ash content of the tibiae and the lowered calcium values of the blood serum. A few birds in Lots 6 and 11, 0.05 per cent fortified cod liver oil and 0.50 per cent corn oil-sterol solution, respectively, exhibited mild symptoms of vitamin D deficiency. The ash percentage of these lots is also slightly below normal. None of the other lots showed any signs of deficiency and their ash values were normal. It is apparent from these results that it required approximately 7 rat units of vitamin D or less per 100 gm. of ration from cod liver oil and between 72 and 143 rat units of sterol origin to promote normal calcification. On account of the apparent greater efficiency of the natural vitamin D of cod liver oil, it required a smaller number of units as irradiated ergosterol for normal calcification when part of the antirachitic activity was derived from the natural source. The ration of Lot 7, containing 3 units from cod liver oil and 20 units from ergosterol, gave ash values comparable to the ration of Lots 2 and 12, which received 7 units from cod liver oil and 143 units from irradiated sterol in corn oil, respectively.

In order to verify the results of the previous experiment and obtain more definite information on the comparative antirachitic efficiency of irradiated ergosterol dissolved in corn oil and cod liver oil and on the

TABLE III

THE COMPARATIVE EFFICIENCY OF VITAMIN D FROM COD LIVER OIL AND IRRADIATED ERGOSTEROL IN CORN OIL AND COD LIVER OIL

Lot No.	Additions to ration	Vitamin D rat units per 100 gm. ration	Average final weight	Blood analysis		Average ash in tibiae
				Ca. per 100 cc. serum	P. per 100 cc. serum	
			gm.	mg.	mg.	per cent
1	None	0	271	7.2	6.8	42.7
2	0.25 per cent cod liver oil	7	357	11.2	8.7	52.1
3	0.50 per cent cod liver oil	14	360	10.9	8.5	51.8
4	1.00 per cent cod liver oil	28	375	11.2	8.5	52.0
5	0.01 per cent ergosterol—cod liver oil solution	(0.3)+ 2	314	9.1	7.6	45.8
6	0.05 per cent ergosterol—cod liver oil solution	(1.5)+10	361	11.4	8.0	49.3
7	0.10 per cent ergosterol—cod liver oil solution	(3) +20	360	11.6	8.7	51.4
8	0.25 per cent ergosterol—cod liver oil solution	(7)+50	376	11.7	8.8	53.3
9	0.01 per cent ergosterol—corn oil solution	1+	229	7.2	5.6	43.1
10	0.25 per cent ergosterol—corn oil solution	36	262	8.5	7.3	42.8
11	0.50 per cent ergosterol—corn oil solution	72	329	10.7	8.6	49.1
12	1.00 per cent ergosterol—corn oil solution	143	343	10.5	8.0	50.3

Note: In Lots 5, 6, 7, and 8 the vitamin D units furnished by the cod liver oil and the irradiated ergosterol are indicated separately. Thus, (3)+20 (Lot 7) implies 3 units from cod liver oil and 20 from the sterol.

minimum number of rat units of the different products that were required for normal calcification in the chick, another experiment was initiated. New samples of cod liver oil and irradiated ergosterol in corn oil were used. Two different strength solutions of irradiated ergosterol in cod liver oil were fed. One represented a 0.01 per cent solution of the sterol and the other a 0.10 per cent solution. Cod liver oil from the same supply as fed unfortified was used in making the solutions. The irradiated ergosterol in corn oil represented a 0.10 per cent solution. The cod liver oil assayed 40 rat units per gram and the irradiated ergosterol 1,666,666 rat units per gram of the sterol. Each lot contained 17 chicks. The solutions were evaporated on fresh portions of ration every 7–10 days.

TABLE IV

THE CALCIFYING VALUE OF VITAMIN D FROM COD LIVER OIL AND IRRADIATED ERGOSTEROL IN CORN OIL AND COD LIVER OIL

Lot No.	Additions to ration, in per cent	Vitamin D rat units per 100 gm. ration	Average final weight	Blood analysis		Average ash in tibiae
				Ca. per 100 cc. serum	P. per 100 cc. serum	
			gm.	mg.	mg.	per cent
1	None	0	182	5.6	6.2	41.8
2	0.05 cod liver oil	2	274	9.6	6.8	46.1
3	0.10 cod liver oil	4	313	10.1	6.8	49.4
4	0.20 cod liver oil	8	297	11.1	7.5	50.2
5	0.30 cod liver oil	12	339	11.4	7.2	51.6
6	0.50 cod liver oil	20	310	10.5	8.4	50.1
7	0.03 *fortified cod liver oil	(1)+ 50	310	9.9	8.1	49.5
8	0.05 fortified cod liver oil	(2)+ 83	310	9.7	8.0	51.3
9	0.10 fortified cod liver oil	(4)+166	290	10.3	8.1	50.0
10	0.20 fortified cod liver oil	(8)+332	317	9.9	7.3	50.1
11	0.03 †diluted fortified cod liver oil	(1)+ 5	289	9.5	7.7	49.7
12	0.05 diluted fortified cod liver oil	(2)+ 8	302	10.1	7.6	50.0
13	0.10 diluted fortified cod liver oil	(4)+ 16	295	10.1	7.9	51.5
14	0.20 diluted fortified cod liver oil	(8)+ 33	314	10.8	7.9	51.6
15	0.03 ‡irradiated ergosterol in corn oil	50	282	9.1	7.6	49.6
16	0.05 irradiated ergosterol in corn oil	83	291	10.5	7.5	50.3
17	0.10 irradiated ergosterol in corn oil	166	289	10.5	7.7	51.1
18	0.25 irradiated ergosterol in corn oil	416	301	10.9	7.7	50.3
19	0.50 irradiated ergosterol in corn oil	833	314	10.9	8.0	50.8

* A 0.10 per cent solution of irradiated ergosterol in cod liver oil.

† A 0.01 per cent solution of irradiated ergosterol in cod liver oil.

‡ A 0.10 per cent solution of irradiated ergosterol in corn oil.

(1)+50=1 unit from cod liver oil and 50 from irradiated ergosterol.

The results summarized in Table IV are self explanatory. All of the chicks that received 0.05 per cent or 2 rat units of vitamin D per 100 gm. of ration from cod liver oil (Lot 2) showed evidence of an antirachitic deficiency; whereas only 3 birds on the 4-unit level (Lot 3) exhibited symptoms of leg weakness. When the amount of cod liver oil was increased to 0.20 per cent, or 8 units, complete protection resulted, as attested by the condition of the birds and the ash content of their tibiae. These findings are in accord with the results of the previous experiment, where 7 rat units of vitamin D per 100 gm. of ration of a different cod liver oil were sufficient for normal calcification. In contrast, it required 83 or more units in the form of irradiated ergosterol in corn oil to produce the same effect. A few

birds on the 50 rat unit ergosterol level (Lot 15) showed visible symptoms of rickets at the sixth week; whereas no evidence of leg weakness and a normal bone ash were observed when 83 or more units of vitamin D were included in the ration. No detrimental or ill effects were noted when an excess or ten times (833 units) the probable minimum requirement of vitamin D were fed as irradiated ergosterol (Lot 19). The feeding of irradiated ergosterol in cod liver oil, either in a 0.01 or 0.10 per cent solution, apparently did not enhance the antirachitic activity of the sterol, because it required considerably more total units of vitamin D in this combination to afford complete protection against rickets than in the form of the untreated cod liver oil. In case of the 0.01 per cent solution of the sterol in cod liver oil it required somewhat more than 2 units from cod liver oil and 8 from activated ergosterol (Lot 12) to give complete protection against rickets. Although the ash content and the blood analysis of this lot appears normal, it was noted that two birds were bordering on leg weakness at 6 weeks. Of the 0.1 per cent ergosterol-cod liver oil solution it required more than 1 unit from the cod liver oil and 50 from the sterol (Lot 7) for maximum calcification.

The results of the foregoing experiment with respect to the comparative effectiveness of irradiated ergosterol in cod liver oil and corn oil, are rather difficult to interpret on account of the increased number of rat units from cod liver oil, irrespective of their greater efficiency, that were fed. To overcome this difficulty cod liver oil from the same supply as used in the former experiment was fortified with such quantities of previously standardized ergosterol that the resulting solution when fed at 0.03 per cent of the ration furnished 5, 10, 25, and 50 rat units of vitamin D per 100 gm. of feed in the form of the sterol in addition to the 1 unit from the cod liver oil. Additional lots on the untreated cod liver oil and the irradiated ergosterol in corn oil were included in the trial.

The results of the blood and bone analysis (Table V) show that it required between 4 and 8 units of vitamin D per 100 gm. of basal ration from cod liver oil to give complete protection; substantiating the results of former trials. Apparently 4 units were very near the minimum requirement, since only two birds showed slight evidence of a deficiency. On the contrary, it required between 75 and 100 rat units of irradiated sterol in corn oil to meet the requirements of the chick. The feeding of irradiated ergosterol in cod liver oil apparently did not influence the antirachitic value of the sterol, since it required 50 or more rat units in the form of ergosterol in addition to the 1 unit supplied by the cod liver oil for normal

TABLE V

THE ANTIRACHITIC EFFICIENCY OF IRRADIATED ERGOSTEROL AND COD LIVER OIL FOR THE CHICKEN

Lot No.	Additions to ration, in per cent	Vitamin D rat units per 100 gm. ration	Average final weight	Blood analysis		Average ash in tibiae
				Ca. per 100 cc. serum	P. per 100 cc. serum	
			gm.	mg.	mg.	per cent
1	None	0	168	5.8	5.9	39.2
2	0.05 cod liver oil	2	261	8.5	5.8	43.3
3	0.10 cod liver oil	4	255	10.3	6.4	49.8
4	0.20 cod liver oil	8	287	12.2	6.4	50.8
5	*0.015 ergosterol in corn oil	25	202	8.4	6.2	43.2
6	0.030 ergosterol in corn oil	50	243	11.0	5.8	48.5
7	0.045 ergosterol in corn oil	75	261	10.6	6.5	49.9
8	0.060 ergosterol in corn oil	100	290	11.1	7.1	50.1
9	0.030 ergosterol in cod liver oil	(1)+ 5	210	8.2	6.4	41.1
10	0.030 ergosterol in cod liver oil	(1)+10	231	8.5	6.5	42.0
11	0.030 ergosterol in cod liver oil	(1)+25	235	9.7	7.4	47.3
12	0.030 ergosterol in cod liver oil	(1)+50	260	10.2	7.1	49.2

* Represented a 0.1 per cent solution of irradiated ergosterol in corn oil.

The cod liver oil solution in Lots 9, 10, 11, and 12 represented 0.01. 0.02, 0.05, and 0.10 per cent solutions of irradiated ergosterol in the oil, respectively.

(1)+50=1 rat unit from cod liver oil and 50 from irradiated ergosterol

calcification. Although there was no evidence of leg weakness in the latter group (Lot 12) the ash values are questionably normal. The results, in general, corroborate the data of the previous experiments.

With the marked difference in the antirachitic value of vitamin D from cod liver oil and irradiated ergosterol, the relative efficiency of irradiated yeast as a source of this factor presented itself. It was also thought advisable to compare the effectiveness of ergosterol prepared from yeast with that prepared from fungus. Accordingly, we proceeded to determine the comparative effectiveness of vitamin D from cod liver oil, irradiated yeast,[1] and irradiated ergosterol of yeast[1] and fungus origin. The products, as in previous experiments, were assayed for anti-rachitic activity on rats prior to the start of the experiment.

The trial included 12 lots of 15 chicks each. One lot was put on the basal ration, two on cod liver oil, three on irradiated yeast, and three on each

[1] We are indebted to Standard Brands, Inc., of New York City, for the irradiated yeast and ergosterol of yeast origin.

sample of irradiated ergosterol in corn oil. To equalize the oil and yeast intake of all lots, 1 per cent of corn oil and 1 per cent of untreated yeast were included in the basal ration; the cod liver oil and irradiated products replacing an equivalent amount of the corn oil or untreated yeast in the basal mixture.

It is evident from the data in Table VI that it required somewhat more than 100 rat units of vitamin D per 100 gm. of feed of either irradiated ergosterol sample for normal calcification. No outstanding difference in the protection afforded by the two different solutions of the irradiated sterol is apparent. Irradiated yeast did not prove so efficient as irradiated ergosterol when fed on an equivalent rat unit basis. This observation is not in accord with the findings of Steenbock, Kletzien, and Halpin (5) who reported that it required from 7.5 to 60 per cent cod liver oil equivalence as irradiated yeast and from 40 to 120 per cent cod liver oil equivalence as irradiated ergosterol for normal bone production. The superiority of the natural vitamin D from cod liver oil is again demonstrated—requiring approximately 8 rat units or less per 100 gm. of ration for normal protection. The blood analyses, in general, approximate the ash determinations.

It should be noted that the lower per cent of tibia ash in this experiment

TABLE VI

THE EFFECTIVENESS OF VITAMIN D FROM COD LIVER OIL AND IRRADIATED ERGOSTEROL AND YEAST FOR CALCIFICATION IN THE CHICKEN

Lot No.	Substitutions in basal ration, in per cent	Vitamin D rat units per 100 gm. ration	Average final weight	Blood analysis		Average ash in tibiae
				Ca. per 100 cc. serum	P. per 100 cc. serum	
			gm.	mg.	mg.	per cent
1	None	0	186	7.8	5.1	37.4
2	0.10 cod liver oil	4	404	9.6	6.2	43.3
3	0.20 cod liver oil	8	473	10.5	7.9	46.9
4	0.02 ergosterol (No. 1) in corn oil	50	272	9.2	6.6	39.8
5	0.04 ergosterol (No. 1) in corn oil	100	332	10.3	6.2	42.0
6	0.08 ergosterol (No. 1) in corn oil	200	390	10.8	7.0	46.9
7	0.015 ergosterol (No. 2) in corn oil	50	320	9.8	6.4	42.9
8	0.03 ergosterol (No. 2) in corn oil	100	331	11.0	6.4	44.5
9	0.06 ergosterol (No. 2) in corn oil	200	393	11.0	6.3	46.1
10	0.15 irradiated yeast, 25 D	50	188	8.5	5.2	38.7
11	0.30 irradiated yeast, 25 D	100	199	9.6	5.2	39.7
12	0.60 irradiated yeast, 25 D	200	313	9.2	6.0	43.8

No. 1 irradiated ergosterol was prepared from yeast, while No. 2 was of fungus origin.

was due to the fact that both cartilages were left intact when the bones were cleaned of adhering flesh, while in the previous experiments the proximal cartilages were removed. We have no explanation for the significantly greater growth obtained in the cod-liver-oil-fed lots. Previous experiments did not show this difference.

Discussion

The data conclusively show that irradiated ergosterol and irradiated yeast are considerably less effective than the rat equivalent amount of cod liver oil for calcification in the chicken. In this regard, the data substantiate the findings of other investigators. However, the results of the present investigation show the irradiated products somewhat more efficient than reported by Mussehl and Ackerson (1), Massengale and Mussmeier (2), and Steenbock, Kletzien, and Halpin (5). The reports of these investigators are rather difficult to interpret in terms of our results, in that they did not make comparisons on a minimum protective basis. In general, they indicate that it required from 40 to 100 times as many rat units of the irradiated products as from cod liver oil, whereas our results only show an approximate 15 to 20 fold increase.

It is interesting to speculate why the chicken is not able to utilize vitamin D from the three sources equally as efficiently as the rat. If it is a question of species difference, are the results due to a difference in amount or degree of absorption of the vitamin, or to an actual difference in the vitamin D substance? Klein and Russell (8) have reported that they were able to recover 26.5 and 43.1 per cent of the total vitamin D units fed to chicks as irradiated ergosterol in corn oil and cod liver oil, respectively. This would suggest that the difference in antirachitic effectiveness is not one of absorption. On the other hand, if the difference in efficiency is one of substance, we must assume, like Steenbock, Kletzien, and Halpin (5), that the natural vitamin D from cod liver oil and irradiated yeast and ergosterol are not identical. Work by English (9) and German (10) investigators shows that there are two forms of vitamin D in ordinary irradiated ergosterol. Accordingly, there is the possibility that one form might prove more efficient than the other in case of the chicken while both might serve equally efficiently for the rat.

Considerable study has been devoted to the effect of vitamin A on the comparative efficiency of cod liver oil and irradiated ergosterol. Poulsson (11) suggested that the vitamin A of cod liver oil was responsible for the superior effect of cod liver oil over ergosterol. Russell and Klein (4) and

Steenbock, Kletzien, and Halpin (5), respectively, on the contrary, found that dried yellow carrots or carotene failed to increase the effectiveness of the irradiated sterol for chicks. Likewise, Hunter, Dutcher, and Knandel (12), who used alfalfa meal as an additional source of vitamin A, report negative results. Apparently vitamin A is not a factor in the comparative antirachitic efficiency of irradiated ergosterol for the chicken.

SUMMARY

1. Irradiated yeast and irradiated ergosterol dissolved in linseed oil, corn oil, or cod liver oil are less effective than the rat equivalent amount of cod liver oil for calcification in the chicken.

2. It required from 15 to 20 times as many rat units of vitamin D per unit of feed in the form of irradiated ergosterol as in the form of cod liver oil for normal bone formation.

3. Cod liver oil did not increase the antirachitic efficiency of irradiated ergosterol for the chicken.

4. Irradiated ergosterol proved somewhat more efficient than irradiated yeast when equivalent rat units of vitamin D were fed.

5. Under the conditions of the experiments, the chick required a minimum of approximately 7 rat units of vitamin D per 100 gm. of ration from cod liver oil for normal calcification.

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LATHYRISM IN THE RAT

By

BEATRICE J. GEIGER, HARRY STEENBOCK,
AND HELEN T. PARSONS*

(From the Departments of Agricultural Chemistry and Home Economics, University of Wisconsin, Madison)

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LATHYRISM, which has been known since the time of Hippocrates, has been very common in the past in India and northern Africa, and small local outbreaks have occurred frequently in Italy, France, and elsewhere in southern Europe (1). For a long time it has been known that the disease is caused by the eating of lathyrus peas of three species, *Lathyrus sativus*, *Lathyrus cicera*, and *Lathyrus clymenum*. After a poor crop of wheat, barley, and other cereals, the poorer people in India and some parts of northern Africa are forced to eat the lathyrus peas as a large part of their diet, and lathyrism then becomes prevalent. The use of the peas as one-third to one-half of the diet for two or three months is considered enough to cause the disease (2). However, not all persons eating such a diet are affected, and in families only certain members are attacked. When the peas are eaten as a smaller proportion of the diet, they are said to be harmless (1).

The outstanding symptom of lathyrism in man is a spasticity and rigidity of the leg muscles which results in a jerky gait with flexed knees, or an inability to walk. The condition develops very rapidly and is often precipitated by exposure to cold, wet, and fatigue. Death seldom results, but there is usually a permanent lameness (1). The symptoms have suggested to various workers the possibility that they may be due to degenerative changes in the spinal cord. In fact Mellanby (3) has gone so far as to suggest that vitamin A may be able to prevent this degeneration. However, he has not reported any experiments to test this idea so far as we are aware.

Different animals are said to vary greatly in their susceptibility to the poisonous action of the lathyrus peas. In Great Britain peas of this type are used as food for farm animals. When mixed in the ration in not too

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large proportion they are said to give satisfactory results, although occasionally bad effects have been reported (1). Of the farm animals, horses have been found to be peculiarly susceptible. Stockman (1) stated that 20 per cent of lathyrus peas in the diet is harmful to horses, while pigs and cattle can tolerate higher levels. Feeding experiments have been carried out using monkeys, rabbits, guinea pigs, rats, pigeons, and ducks. According to Stockman (4) monkeys have spasmodic attacks of a dramatic kind occurring at irregular intervals, in addition to the paralysis. He reported that death was due to failure of the respiratory muscles. In his experiments on rabbits, in which he used seven animals, he observed in some cases a very transient and slight spasmodic condition of the legs, but most of his animals grew normally. He assumed the presence of a toxin and inferred that rabbits are susceptible to the toxin, but that in ordinary feeding experiments they get too little poison to produce symptoms. In four out of five guinea pigs he noted tremors, spasticity, and weakness of the legs and reported that all died in 8 to 35 days. He stated that "the lethal action is not a specific lathyrus action, but seems to be due to the fact that the proteins of lathyrus peas cannot be assimilated and utilized by guinea pigs." McCarrison (5) reported that he was unable to cause lathyrism in rats even when feeding lathyrus peas as the only food. Zagami (6) also failed to secure symptoms characteristic of lathyrism in rats. He fed a diet made up entirely of lathyrus peas for 150 days. He did report a decrease in the rate of gain, retardation in sexual development, and a decrease in calcification of the skeleton, which can be readily understood, inasmuch as all seeds when fed by themselves are seriously deficient in various nutritive elements. Acton is said to have produced paralysis in ducks by feeding them lathyrus peas (1). Pigeons have been reported insusceptible to the toxin (4).

The discrepancies in the results of different investigators are difficult of interpretation. Undoubtedly many of them were due to the use of poor basal diets which frequently were deficient in mineral elements, vitamin D, and good protein, and probably were otherwise poorly constituted. Mellanby (3), for instance, believes that vitamin A furnished variable protection depending on the amount present. Emphasis should, however, also be placed on the fact that the investigators frequently used different species and varieties of lathyrus peas and no mention was made of the conditions under which they were grown.

There is a difference of opinion among experimenters as to the nature of the toxic substance in the lathyrus peas. Stockman (7) asserted that

the toxic principle was an alkaloid which he obtained in very small amount from the peas and with which he could produce paralysis in frogs, monkeys, and mice. However, Acton and Chopra insisted that an amine and not an alkaloid was the toxic substance (cited from [1]). These investigators reported paralysis in the hind legs of monkeys, guinea pigs, rats, and mice with the pure hydrochloride of the amine obtained from the peas. In contrast to both of these views, Anderson, Howard, and Simonsen (8) are of the opinion that the poisonous effect was due to other seeds, especially *Vicia sativa* variety *Angustifolia*, found mixed with the lathyrus peas. They observed no toxic effects of any kind when botanically pure *Lathyrus sativus* seeds were fed to animals, but stated that when seeds of *Vicia sativa* were fed to monkeys, nervous and muscular symptoms resulted, and when fed to ducks they caused death. *Vicia sativa* was reported as containing bases showing alkaloidal properties of which they isolated three, vicine, divicine, and a cyanogenetic glucoside, vicianin, at least one of which, divicine, produced characteristic symptoms when injected into guinea pigs. A still different theory of the cause of lathyrism was advanced by Young (2) after he made a field study of the disease in India. He suggested that the disease was due primarily to a deficiency of vitamin A.

From the preceding it is evident not only that there is considerable difference of opinion regarding the etiology of lathyrism, but also that the properties of different species are very different and hardly specific.

In the experiments detailed in the following report the effect of feeding lathyrus peas to rats was determined. An attempt was made to ascertain whether the abnormal condition produced by the peas was ameliorated or prevented by the feeding of cod liver oil or yeast as vitamin supplements. The species of lathyrus peas which was used in our experiments was *Lathyrus odoratus*, the sweet pea used for ornamental purposes in this country.

EXPERIMENTAL

The composition of the diets is shown in Table I.[1] The casein was a commercial product freed from vitamin A by heating it at 90°C. for two weeks. The dextrin in reality represented cooked starch. It was prepared by mixing enough water with cornstarch to make lumps, autoclaving it for 2

[1] We wish to thank John Bodger and Sons of El Monte, California, for the sweet peas, E. R. Squibb and Sons for the cod liver oil, and National Oil Products Company for the cod liver oil concentrate used in these experiments.

TABLE I

COMPOSITION OF DIETS*

Lots	1	3 26 73 78	4	27 39 50 74	28†	29†	51 75	52 76	55	57	77	79	81	82	83	117‡	121§
Sweet peas	—	50	80	50	50	50	25	25	50	50	50	—	12.5	5	50	—	50
Casein (Vitamin A free)	10	10	10	10	10	10	10	10	10	10	—	18	14	14	—	18	10
Salts 40‖	4	4	4	4	4	4	4	4	4	4	—	4	4	4	—	4	4
Dextrin	34	36	6	34	36	34	61	59	33.9	26	—	68	63.5	71	—	71	35
Cod liver oil	2	—	—	2	—	2	—	2	2	—	—	2	—	—	2	1	1
Cod liver oil concentrate	—	—	—	—	—	—	—	—	0.1	—	—	—	—	—	—	—	—
Yeast	—	—	—	—	—	—	—	—	—	10	—	8	6	6	—	6	—
Garden peas	50	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Yellow corn	—	—	—	—	—	—	—	—	—	—	26	—	—	—	24	—	—
Wheat gluten	—	—	—	—	—	—	—	—	—	—	20	—	—	—	20	—	—
$CaCO_3$	—	—	—	—	—	—	—	—	—	—	3	—	—	—	3	—	—
NaCl	—	—	—	—	—	—	—	—	—	—	1	—	—	—	1	—	—

* All rations except 78 contained 50 mgs. irradiated yeast per 100 gms. of ration.

† Rations 28 and 29 were made up with peas which had been cooked for 2½ hours.

‡ Ration 117 had the water extract from 100 gms. of sweet peas evaporated on 71 parts of dextrin.

§ Ration 121 contained sweet peas which had been boiled and thoroughly extracted with hot water.

‖ Steenbock, H., and Nelson, E. M., *Jour. Biol. Chem.*, 1923, **66**, 355.

hours at 15 lbs. pressure, drying it, and then grinding. The cod liver oil concentrate was incorporated in the ration by dissolving it in ether, pouring the ether solution on part of the dextrin and then evaporating off the solvent in a current of air. This was then mixed with the other ingredients of the ration. The yeast was obtained from the Northwestern Yeast Company and had been previously tested in this laboratory for its vitamin B content. The irradiated yeast was yeast which had been irradiated for 2 hours with an Alpine Sun Lamp at a distance of 18 inches with stirring.

The rations were so outlined as to be adequate in all respects, except that in some, vitamin A was absent aside from such amounts as were contained in the sweet peas themselves. For control purposes to demonstrate the general adequacy of the supplements, garden peas were substituted for the sweet peas at the higher levels and 2 per cent of cod liver oil was included. With these in the diet approximately normal growth was obtained and the animals maintained themselves in excellent condition for 14 weeks. At this time the trials were terminated. The rations were fed *ad libitum*, the food consumption being recorded during the first 4 or 5 weeks of the experiments. Male albino and piebald rats weighing between 50 and 60 grams when 3 to 4 weeks old were used in lots of 4. Each rat was kept in a separate cage with a raised screen bottom. Distilled water was given in addition to the ration. The animals were weighed once a week.

Contrary to the findings of McCarrison (5) and Zagami (6) we have succeeded in inducing lathyrism in rats. Very little growth occurred on the diets which contained 80 per cent of sweet peas and the rats lived for only 4 to 6 weeks (Lot 4). As the percentage of sweet peas in the diet was decreased, greater growth resulted. Of the 40 animals receiving 50 per cent of sweet peas from the time of weaning, 3 grew to a weight of 160 grams, during 3-1/2 months on the ration; the rest weighed less than this. One of the 8 animals on the 25 per cent level attained a weight of 215 grams. However, when the sweet peas were fed as 12.5 per cent of the ration or less, practically normal growth was secured for 20 weeks in a group of 8 rats. The growth of the animals is summarized in Table II.

Depression of growth was only one effect of the diets containing sweet peas. The first sign of abnormality appeared after about 4 weeks on diets containing 50 per cent of sweet peas and after 6 weeks on those containing 25 per cent. All the animals developed a marked spinal curvature. The long bones were variously deformed, and the rat frequently walked with a shambling gait. The abnormalities became progressively worse and apparently the animals were in discomfort, as they became very inactive.

TABLE II

GROWTH OF RATS ON SWEET PEA DIETS

(Average weights)

Lot No.	Character of ration	Weight					Remarks
		Initial	at 4 weeks	at 6 weeks	at 10 weeks	at 15 weeks	
1	50% garden peas+2% C.L.O.	gms. 56	gms. 113	gms. 136	gms. 168	gms.	Control group. Approximately normal growth. In excellent condition when etherized after 13 weeks.
4	80% sweet peas	52	75				Very little growth. Digestive disturbance (flatulence, diarrhea). Respiratory difficulty after 3 weeks. Hind legs of 2 rats paralyzed after 5 weeks. All died during 5th or 6th week.
3	50% sweet peas	54	104	112	123		Slow growth for 4 to 6 weeks. Hind legs lame after 4 weeks, those of 2 rats in Lot 3 almost completely paralyzed. Lordosis in thoracic region. Sternum misshapen in 2 rats. Some digestive disturbance. 3 died between 7th and 12th weeks, others etherized.
26	50% sweet peas	55	111	123	118		
27	50% sweet peas +2% C.L.O.	55	103	125	143		Some improvement in growth with addition of C.L.O. Lameness and lordosis after 4 or 5 weeks. Hind legs partially or completely paralyzed. All rats in Lot 27 developed hernias. Ventral or lateral spinal curvature in all. 4 died between 5th and 12th weeks, others etherized.
39	50% sweet peas +2% C.L.O.	55	85	114	143		
50	50% sweet peas +2% C.L.O.	58	107	132	143		
55	50% sweet peas +2% C.L.O.+ 0.1% C.O.C.†	54	101	113			No better growth than Lots 27, 39 and 50. Lameness and lordosis after 5 weeks. One rat developed a hernia. Etherized after 7 weeks.
57	50% sweet peas +10% yeast	57	121	132	127		No better growth than Lots 3 and 26. Symptoms of lathyrism after 5 weeks. All died between 6th and 15th weeks.

† C.O.C. = Cod liver concentrate.

TABLE II (*Continued*)

Lot No.	Character of ration	Weight					Remarks
		Initial	at 4 weeks	at 6 weeks	at 10 weeks	at 15 weeks	
		gms.	gms.	gms.	gms.	gms	
51	25% sweet peas	54	91	118	158	168	Better growth than with 50%. Growth fairly good for 12 weeks with no C.L.O. in diet. Symptoms of lathyrism after 6 weeks. Etherized after 18 weeks.
52	25% sweet peas +2% C.L.O.	54	89	112	159	176	Slightly better growth than Lot 51. Symptoms of lathyrism in 3 rats after 6 weeks, in one after 9 weeks. Etherized after 18 weeks.
81	12.5% sweet peas	53	139	185	249	277	Normal growth with no source of vitamin A except sweet peas. No symptoms of lathyrism after 20 weeks. Yeast included after 5 weeks to supplement vitamin B in sweet peas since appetite was declining. Etherized after 20 weeks. Slight enlargement of costochondral junctions on autopsy.
82	5% sweet peas	57	117	182	208	242	
28	50% sweet peas (cooked)	52	92	100	100		Same results as with uncooked sweet peas. 5 died between 5th and 12th weeks, others etherized after 12 weeks.
29	50% sweet peas (cooked) +2% C.L.O.	55	102	117	135		
73	50% sweet peas	359	345	348	347	350	Weight maintained. Lameness appeared in all rats. Etherized after 15 weeks.
74	50% sweet peas +2% C.L.O.	343	340	352	351	346	Slight increase in weight. No lameness developed. Etherized after 15 weeks.
75	25% sweet peas	383	363	372	366	377	Weight maintained. No symptoms of lathyrism. Etherized after 15 weeks.
76	25% sweet peas +2% C.L.O.	342	337	346	347	349	Slight increase in weight. No symptoms of lathyrism. Etherized after 15 weeks.

TABLE II (*Continued*)

Lot No.	Characte rof ration	Weight					Remarks
		Initial	at 4 weeks	at 7 weeks	at 10 weeks	at 15 weeks	
78	50% sweet peas (no vitamin D.)	51	90	111	126		Slightly poorer growth than Lots 3 and 26 which received irradiated yeast. All died between 3rd and 14th weeks.
77	#2965+50% sweet peas	54	91	88	79		Lathyrism after 3 weeks. Six died between 6th and 14th weeks, others etherized.
83	#2965+50% sweet peas+ 2% C.L.O.	55	82	93	102		
79	Control Diet	53	146	207	256		Normal growth. Etherized after 11 weeks.
117	Water extract of sweet peas	54	135	129	134		Good growth for 4 weeks, then decline. Lathyrism after 4 weeks. All died between 7th and 12th weeks.
121	50% water extracted sweet peas	54	73	116	203		Good growth. No symptoms of lathyrism after 11 weeks. Experiment continuing.

In a few cases the hind legs were completely paralyzed. In 2 cases the front legs were also involved, the feet turning out at the wrists. In many of the animals the sternum was deformed. For a typical illustration of such animals see Plate 1.

Although hernia in the rat is a very unusual phenomenon, practically never occurring in the stock colony and to the best of our knowledge never having been reported in relation to dietary abnormality in the rat, it occurred in 10 cases, or 25 per cent of the young animals receiving the 50 per cent level of sweet peas. Some of the hernias were permanent while others appeared and disappeared at intervals. The contents of the hernias varied, sometimes consisting of the cecum and part of the intestines only, while in others a kidney, a testis, and even the stomach and spleen protruded in addition.

The severity of the skeletal changes became evident on autopsy. The spinal column in every case showed extreme curvature in the thoracic

region. In most of the animals the curvature was only ventral although it sometimes was also lateral. Invariably the ventral curvature involved

PLATE 1.—A side view of one rat from Lot 3 showing the spinal curvature characteristic of animals on diets containing 50 and 25 per cent of sweet peas.

the lower seven thoracic vertebrae. An outstanding feature was the short radius which the curvature assumed even in the larger animals. This did

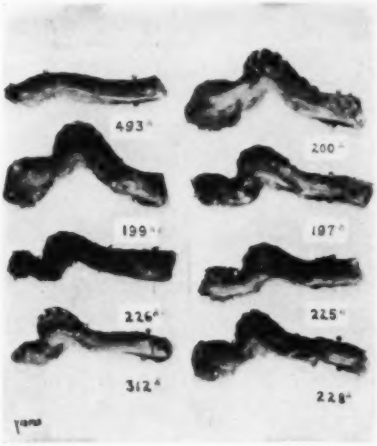

PLATE 2.—Photographs of vertebral columns showing curvatures in the thoracic region. These photographs were taken with the ventral side uppermost and the cervical end to the left. Rat 493—a normal animal from the stock colony. Rats 197, 199, 200 from Lot 30 received a diet containing 50 per cent of sweet peas and 2% of cod liver oil. Rats 225, 226, 228 from Lot 57 received a diet containing 50 per cent of sweet peas and 10 per cent of yeast. Rat 312 from Lot 78 received 50 per cent of sweet peas.

not exceed 3/8 of an inch (Plate 2). In most cases there was also a dorsal curvature in the lumbar region. In some rats the tail made an acute angle with the body.

Although the spinal curvature dominated the picture the thoracic cavity was otherwise affected. In the first place the sternum was frequently deformed. Sometimes it assumed a dorsal-ventral curvature, but in other cases it was also deformed laterally. It was noted that in addition the ribs were frequently misshapen. Sometimes the costochondral junc-

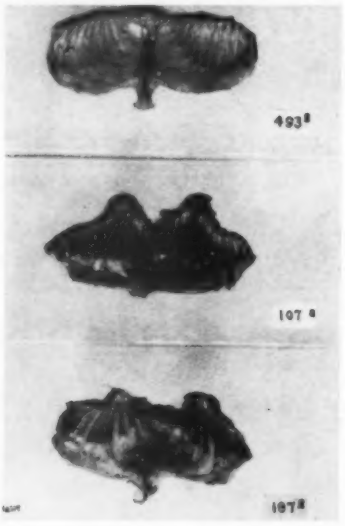

PLATE 3.—Photographs showing malformation of ribs and sternums. Rat 493—A normal animal from the stock colony. Rats 107 and 197—From Lots 27 and 50 respectively received a diet containing 50 per cent of sweet peas and 2 per cent of cod liver oil. Note angulation at the costochondral junctions and deformation of the sternum.

tions were merely enlarged, but in other cases angulation was also pronounced. The photographs shown in Plate 3 may be taken as typical examples. It was evident that these changes could not have been caused by rickets, since all the rations contained 4 per cent of a complete salt mixture as well as vitamin D in an amount previously shown in this laboratory to be ample for protection.

The femora and humeri were badly deformed. The upper ends of the femora were broadened and rough areas were present on either side at the points of attachment of the muscles. The humeri were also thickened and

showed similar roughened areas (Plate 4). Taking an average of 30 animals and comparing them with a similar number which had similar weights recorded by Donaldson (9), the weights of femora and humeri were found to be 33 and 26 per cent less, respectively.

In view of the deformities both in the vertebral column and in the long bones it was believed possible that changes in mineralization of the bones had occurred. However, that this was not extreme was evident from the fact that the bones were observed to be neither brittle nor flexible. Deter-

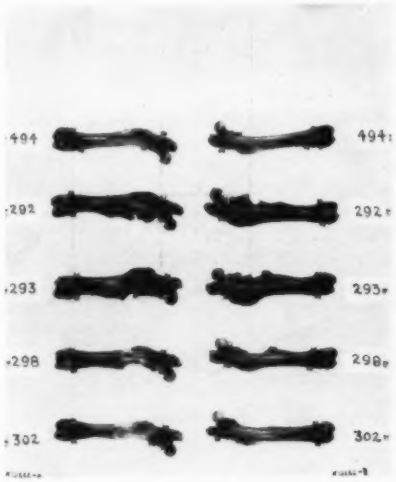

PLATE 4.—Photographs of femora from adult rats which had received diets containing 25 and 50 per cent of sweet peas for 16 weeks after they had attained a weight of 300 to 400 grams. The femora at the left show the appearance of the dorsal surface of the bones while those on the right show the ventral surfaces.

Rat 494—A control animal from the stock colony. Rat 292 from Lot 73 received 50 per cent of sweet peas. Rat 293 from Lot 74 received 50 per cent of sweet peas and 2 per cent of cod liver oil. Rat 298 from Lot 75 received 25 per cent of sweet peas. Rat 302 from Lot 76 received 25 per cent of sweet peas and 2 per cent of cod liver oil.

Note the abnormal shape of the bones and the exostoses at the points of attachment of the muscles. The bones of the animals which received 25 per cent of sweet peas are less abnormal than those on the 50 per cent level. The addition of cod liver oil to the rations prevented the bone changes to some extent.

minations of ash were made in the usual manner. The bones were dissected out immediately after death, extracted with alcohol and ether, dried, and ashed. The results are presented in Table III. It will be noted from the

table that the ash content of the bones of the adults falls within the normal limits. In the young both the amount of ash and percentage of ash were decreased.

TABLE III

PERCENTAGE OF ASH IN THE BONES OF RATS ON SWEET PEA DIETS

Lot	No. of femora	Sweet pea additions	Average age	Average weight	Weightof ether extracted femur	Weight of ash	Ash
			days	gms.	gms.	gms.	%
26, 28	3	50%	113	116	0.317	0.168	52.8
27, 29, 39	6	50%+2% C.L.O.	100	136	0.337	0.177	52.8
55	2	50%+2% C.L.O.+ 0.1% C.O.C.†	77	116	0.287	0.149	52.0
57	1	50%+10% yeast	114	107	0.299	0.173	58.0
51	1	25%	161	198	0.530	0.336	63.3
52	3	25%+2% C.L.O.	156	209	0.471	0.293	62.1
73	2	50%	274	354	0.651	0.400	61.8
74	2	50%+2% C.L.O.	281	336	0.687	0.420	61.0
75	2	25%	247	355	0.631	0.394	62.5
76	2	25%+2% C.L.O.	257	340	0.635	0.416	65.5
Stock	15	Stock ration only	101	294	0.455	0.283	62.2
Stock	14	Stock ration only	201	360	0.544	0.338	61.9

† C.O.C. = Cod liver oil concentrate.

Inasmuch as the blood phosphorus and calcium relations have served as valuable indices of the state of bone metabolism, such data on our animals were obtained. The Fiske-Subbarow method (10) was used for determination of inorganic phosphorus. The serum calcium was determined on the protein-free filtrate obtained after treating the serum with four times its volume of 10 per cent trichloracetic acid. Aliquots corresponding to 1 cc. of serum were used. The hydrogen ion concentration was adjusted in the manner used by McCrudden (11) for organic materials. The calcium was precipitated with ammonium oxalate and after treatment with normal H_2SO_4 was titrated with N/100 $KMnO_4$, using a micro-burette. The values obtained are tabulated in Table IV. Inspection of the collected data on the adults reveals that the serum calcium and inorganic phosphorus were within the normal limits. Unfortunately blood analyses on young rats were not made.

One of the outstanding changes in the bones was their congested appearance. Distinct hemorrhages were also noted macroscopically in the

muscle and connective tissue immediately contiguous to the roughened areas on some of the femora and humeri. The appearance of the bones themselves suggested a possible stimulation of hematopoietic activity. Individual determinations of hemoglobin by the Newcomer method, using blood obtained from the tails of 23 of the animals, gave results ranging from 12.2 to 15.9. Three-fourths of the values fell above 14, which in-

TABLE IV

MILLIGRAMS PER CENT OF CALCIUM AND PHOSPHORUS IN THE BLOOD OF RATS ON SWEET PEA DIETS

Lot	No. of rats	Sweet pea additions	Serum Ca	Serum P
			mg. %	mg. %
73	4	50%	10.4	6.9
74	4	50%+2% C.L.O.	11.2	7.0
51	4	25%	10.0	9.1
75	3	25%	9.9	6.9
76	4	25%+2%C.L.O.	10.0	6.6
52	4	25%+2% C.L.O.	10.2	9.6

dicates a tendency toward a high level. The erythrocyte count of these same animals estimated in a Levy counting chamber with single Neubauer ruling averaged 9,900,000 with a range of 7,800,000 to 12,000,000. All but 3 counts were above 9 million and 10 were above 10 million, thus falling in the upper part of the normal zone or slightly above. Averaged values of three to four animals in each group are shown in Table V. Examination of the spleens showed them to be somewhat smaller in pro-

TABLE V

HEMOGLOBIN VALUES AND ERYTHROCYTE COUNTS OF RATS ON SWEET PEA DIETS

Lot	No. of rats	Sweet pea additions	Hemoglobin, gms. per 100 cc. of blood	Erythrocytes per cu. mm. of blood
73	4	50%	15.48	9,863,000
74	4	50%+2% C.L.O.	14.75	10,163,000
51	4	25%	14.20	8,850,000
75	3	25%	14.02	10,850,000
76	4	25%+2% C.L.O.	13.55	10,330,000
52	4	25%+2% C.L.O.	14.26	9,300,000

portion to the body weight, especially in those animals receiving the 50 per cent level of sweet peas (Table VI).

TABLE VI

THE WEIGHT OF THE SPLEEN OF RATS ON SWEET PEA DIETS

Lot	No. of rats	Sweet pea additions	Range of body weight	Average spleen weight	Average body weight divided by average spleen weight
			gms.	gms.	
73	4	50%	294–400	0.740	475
26	2	50%	125–147	0.270	507
27	1	50%+2% C.L.O.	147	0.311	471
29	1	50%+2% C.L.O.	140	0.367	381
50	4	50%+2% C.L.O.	123–151	0.251	549
55	4	50%+2% C.L.O.+0.1% C.O.C.†	109–120	0.589	243
57	3	50%+10% yeast	99–138	0.372	343
75	3	25%	351–427	0.624	607
51	4	25%	130–198	0.461	375
76	4	25%+2% C.L.O.	313–384	0.731	458
52	4	25%+2% C.L.O.	163–215	0.683	233
79	3	Control diet	186–400	0.683	398

† C.O.C. = Cod liver oil concentrate.

To supplement our observations on young animals a few experiments were carried out on old rats. They were put on the ration at a weight of 300 to 400 grams. Some of the gross effects obtained with the young animals were secured with such adults. The femora and humeri became markedly congested, the muscular attachments were hemorrhagic and the cortex was roughened. All the long bones were deformed. Lameness became incident after 12 weeks in the animals which received 50 per cent of sweet peas with no vitamin supplement. The essential differences were that the spinal columns, the ribs, and sternums were normal. Hernia also did not develop.

To pave the way for further studies on lathyrism, but not in direct relation to our immediate problem, an attempt was made to inactivate the toxic principle in the sweet peas by cooking them for 2-1/2 hours. However, no decrease in the severity of the toxic effect could be noted when the cooked peas together with the water in which they were cooked were fed at the 50 per cent level. Later the toxic principle was found to be water soluble and fairly stable to heat at the boiling point of water. In these

experiments the peas were soaked over night in distilled water and were then brought to the boiling point. After standing for 30 minutes the water was poured off. The peas were then covered again with distilled water and the extraction was repeated as before, five times. A final extraction was made with a large amount of water, this extract being discarded. The other combined extracts were concentrated to a small volume at 60 to 65° C. before an electric fan and were then dried on dextrin at the same temperature. When this was incorporated in the ration so that each 100 grams of ration carried an amount of extract equivalent to 100 grams of sweet peas, the same toxic effects were observed as when 50 per cent of sweet peas were fed. Furthermore, the extracted sweet peas fed at the 50 per cent level produced no apparent toxic effects after 11 weeks.

An attempt to prevent or correct the symptoms of lathyrism was made along the lines suggested by Mellanby (3) by vitamin additions. Cod liver oil did not protect the young animals from the toxic effect of the higher levels of sweet peas. In fact with 0.1 per cent of cod liver oil concentrate in addition to the 2 per cent of cod liver oil no decrease in the severity of the skeletal changes could be noted. Dried yeast was also without protective effect. However, the adult animals were protected to some extent by 2 per cent of cod liver oil at both the 50 and 25 per cent levels, which was apparent in the condition of both the femora and humeri. The bones of the animals receiving the cod liver oil were smaller, had fewer exostoses, and were more nearly of the same color as the controls.

It is noteworthy that so much growth was possible when no vitamin A was present in the diets other than that furnished by the sweet peas. With only 5 per cent of sweet peas in the diet normal growth was secured for 20 weeks. This indicates that the sweet peas used by us were unusually rich in vitamin A as compared with other seeds.

It is possible that the vitamin A in the sweet peas exerted a substantial neutralizing effect even without the addition of cod liver oil. It is suggested that future studies designed to determine the neutralizing effect of vitamin A be carried out with water extracts inasmuch as these can be readily obtained free from vitamin A. However, we believe that even with our present technic we discovered some prophylactic action with cod liver oil. The results seemed to be clean-cut with our adult animals.

SUMMARY

Lathyrism was produced in both young and adult rats by feeding diets which contained *Lathyrus odoratus*, the flowering sweet pea.

Growth of the young animals was retarded by sweet peas when they were fed as 80 per cent, 50 per cent and 25 per cent of the diet. However, normal growth was obtained for 20 weeks with 12.5 per cent and 5 per cent of sweet peas. Other symptoms of lathyrism noted were lameness, spinal curvature, sternal curvature, enlargement of the costochondral junctions, and malformation and abnormal red color of the long bones. Calcification was interfered with in young animals. The decided reddish color of the bones harmonized with a slight polycythemia and increased hemoglobin content of the blood. Hernias were also observed.

The young animals received no protection against the toxicity of the sweet peas from cod liver oil or cod liver oil concentrate, or from dried yeast. However, the adult animals were protected to some extent by the inclusion of 2 per cent of cod liver oil in the ration.

Cooking the sweet peas for 2-1/2 hours did not destroy their toxicity.

The toxic factor was extractable from the peas by water at the boiling point.

The sweet peas used by us proved to be a better source of vitamin A than any other seeds previously studied, since normal growth was secured on a diet in which 5 per cent of sweet peas was the only source of vitamin A.

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THE VITAMIN CONTENT OF LICHENS*

By

N. R. ELLIS, L. J. PALMER, AND G. L. BARNUM

(From the Animal Husbandry Division, Bureau of Animal Industry, and the Bureau of Biological Survey, United States Department of Agriculture, Washington)

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THE native forage available to the herds of reindeer and musk-oxen maintained in the arctic regions of North America is by necessity limited chiefly to those plants which grow in the regions bordering on or beyond the arctic circle. The lichens are probably the most important group of plants available for grazing, particularly in the inland areas and during the winter season.[1] With the growth of the herds of reindeer and musk-oxen in Alaska has come the question of finding and selecting the best feeding areas for the herds. Out of these considerations of management has arisen the question of the nutritive value of the lichens. Recent studies (2) on the digestibility of feed by reindeer and musk-oxen have shown that the lichens yield moderately high digestibility coefficients.

An examination of the literature disclosed little or no information on vitamin values of lichens. On the other hand, Hadwen and Palmer (1) have described a number of diseases common in herds of reindeer, certain of which are suggestive of vitamin deficiencies. Dermatitis, keratitis, rickets, and other bone deformities are among those of possibile dietary origin.

THE LICHEN SAMPLES

The estimations of vitamin content have been confined to the vitamin A, vitamin B-complex (with partial differentiation of the B and G factors),

* This study has formed a part of a coöperative investigation on nutrition problems relating to reindeer and musk-oxen by the Bureaus of Biological Survey and Animal Industry of the United States Department of Agriculture. The present study on the vitamin content of lichens was suggested by the Bureau of Biological Survey and the samples were obtained by L. J. Palmer in charge of the reindeer experiment station and grazing investigations of the Bureau of Biological Survey in Alaska. The work was conducted by the other authors named in the nutrition laboratories of the Bureau of Animal Industry at Beltsville, Maryland.

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[1] Hadwen and Palmer (1) have described the grazing conditions and the types of vegetation available in Alaska. They have also compiled a check list of Alaskan range plants.

and the vitamin D factors. Samples of lichens were supplied in 1929 and again in 1930 for the feeding tests. Two compositive samples, designated as Series 1, were made up from samples of separate species for feeding tests during 1929. One sample comprised those species of short lichens which grow in dry areas and the other, the tall forms, from moist areas. The list of species follows:

Short growth type or dry site lichens	Tall growth type or moist site lichens
Alectoria nigricans	*Cetraria islandica*
Alectoria ochroleuca	*Cetraria cucullata*
Cetraria nivalis	*Cladonia alpestris*
Cetraria hiascens	*Cladonia gracilis elongata*
Cladonia bellidiflora hookeri	*Cladonia uncialis*
Cladonia gracilis dilatata	*Cladonia amaurocraea forma*
Lobaria linita	*Cladonia sylvatica sylvestris*
Nephroma arcticum	
Parmelia physodes	
Peltigera species	
Stereocaulon tomentosum	
Thamnolia vermicularis	

Series 2 consisted of an additional pair of samples of short and tall lichens gathered at the experiment station at College, Alaska, in May, 1930. The sample of short growth lichens consisted largely of *Cladonia bellidiflora hookeri* and *Cladonia gracilis dilatata*. The other was made up of mixed species of tall growth lichens as in Series 1. The lichen samples were finely ground in order that the required amounts could be intimately incorporated into the basal diets. Both series were used in the tests on vitamin A and on the vitamin B-complex, while Series 2 was used in the tests reported on vitamin D.

EXPERIMENTAL PROCEDURE

The Growth of Rats on Lichens-Containing Diets

In preliminary tests it was found that rats did not readily consume large amounts of lichens when fed *ad libitum* separately from the basal diet. Incorporation of the lichens into a mixed diet permitted adequate food consumption when the short lichens were used, but not when the tall lichens were present at levels of 10 or 20 per cent. Results of these feeding tests are illustrated in the lower part of Figure 1. The diets were similar to those

used in the vitamin A and the vitamin B-complex tests with the exception that both cod liver oil and yeast were included in the diet. The short lichens

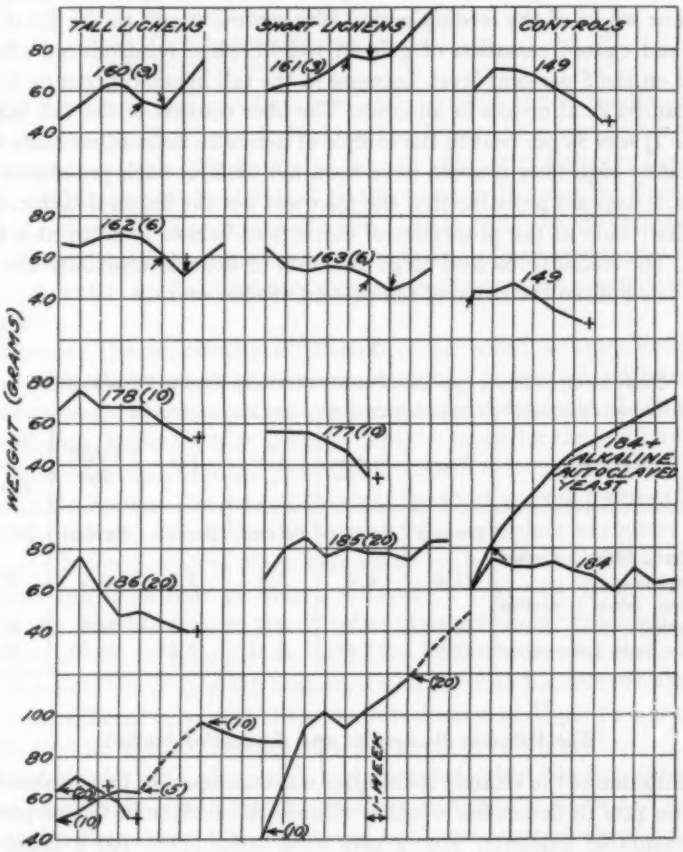

FIGURE 1.—The growth of representative animals used in the tests for the vitamin B-complex, vitamin B, and vitamin G are shown in this figure. At the right are the control animals on basal diets 149 and 184. The middle column shows the animals on diets of short growth lichens while the column to the left shows those animals on diets of tall growth lichens. The diet numbers with percentage of lichens in the diet given in parenthesis are shown for each curve. The growth curves at the bottom of the figure show the effect of the introduction of lichens in diets which contained mineral mixture, yeast, and cod liver oil, along with protein, fat, and carbohydrate sources. The symbols employed are as follows: + death of the animal; ↗ addition of alkaline autoclaved yeast; ↓ addition of rice polish; ← the point where a given level of lichens was introduced into the diet.

at 10 and 20 per cent levels did not lower the food consumption or the rate of growth to any marked extent. In the case of the tall lichens, 5 per cent did not interfere with growth, while 10 per cent retarded growth during the first four weeks of the feeding period (the second month in the life of the rats) and caused cessation of gain in weight when reintroduced after a period on the 5 per cent level. Increase in the tall lichen content to 20 per cent caused fatal results in all cases. The fiber content of the tall lichens (Table I) was 34 per cent in the sample of Series 2. Since other feeds with an equally high fiber content have been fed without such pronounced ill effects, it appears probable that the fiber was not the harmful factor. Further discussion of the properties of extracts of lichens is given at a later point. The inability to feed large amounts of lichens, especially the tall form, has limited the scope of the present studies on rats.

TABLE I

COMPOSITION OF SHORT AND TALL FORMS OF LICHENS USED IN FEEDING TESTS

Type of lichen	Water	Protein	Ash	Fat	Crude fiber	Nitrogen free extract
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
Short form, Series 1 average (5 species)	13.4	6.0	3.4	3.7	15.4	57.9
Tall form, Series 1, average (8 species)	12.5	2.1	1.8	2.3	32.3	49.1
Tall form, Series 2, as received	12.09	2.45	2.57	2.64	34.00	46.25

The Vitamin B-complex and Associated Factors

Estimation of the vitamin B-complex was accompanied by supplemental tests on rats to determine whether vitamin B or vitamin G was present in measurable amounts. Young rats were individually fed a basal diet deficient in the vitamin B-complex. This diet (No. 149) consisted of: water and alcohol extracted casein, 20 per cent; dextrinized rice starch, 65 per cent; lard, 10 per cent; agar, 1 per cent; mineral mixture (Osborne and Mendel), 4 per cent; and cod liver oil fed separately in 0.2 cc. doses three times per week.

Following a short depletion period of one to two weeks on this diet, the lichens were incorporated into the basal diet by substitution of equal amounts of dextrin as follows:

	per cent
Diet 160—tall lichens, Series 1	3
Diet 161—short lichens, Series 1	3
Diet 162—tall lichens, Series 1	6
Diet 163—short lichens, Series 1	6
Diet 177—short lichens, Series 2	10
Diet 178—tall lichens, Series 2	10

The growth curves of selected animals, typical of the results on each diet, are shown in Figure 1. Neither the 3 nor the 6 per cent levels of lichens was capable of maintaining growth any better than the basal diet (No. 149) known to be deficient in the vitamin B-complex. Even when autoclaved yeast,[1] a source of vitamin G, was added to the daily diet of the rats on the diets containing lichens their decline in weight continued, due, evidently, to the absence of even moderate or small amounts of vitamin B.

However, the substitution of 0.5 gram of rice polish for autoclaved yeast resulted in the resumption of gains. Although rice polish contains only small amounts of vitamin G as compared to its rich supply of vitamin B, observations indicated that the growth of the rats after the addition of the 0.5 gram dose was due to the presence of vitamin G in the rice polish rather than in the lichens. Ten per cent levels of the short and tall forms of lichens in Diets 177 and 178, respectively, were still inadequate as sources of the vitamin B-complex since three animals on each diet died within approximately the same time as control animals on Diet 149. Death occurred earlier on Diet 178 than on the complete diets previously described.

Tests on the vitamin G content of the lichens were also made by the use of a diet (No. 184) which contained white corn as a source of vitamin B. This diet differed from diet 149 in the substitution of 25 per cent of ground white corn for the lard and agar and for 14 per cent of dextrin.

Rats on this diet alone and supplemented with alkaline autoclaved yeast as a source of vitamin G are shown in Figure 1. Diets 185 and 186 contained 20 per cent of the short and tall types respectively of lichens of Series 2 in place of an equal percentage of dextrin. Growth curves of rats on these diets indicated that little, if any, vitamin G was present even in the small lichens.

Further evidence that the tall lichens were deficient in the vitamin B-complex was afforded by feeding tests on an extract prepared from the

[1] The yeast was adjusted to a pH of 9.0 with sodium hydroxide, autoclaved for two hours, then neutralized with hydrochloric acid and dried at room temperature with a fan. The daily dose was 0.4 gram.

residue after extraction of the lipids for vitamin A tests (see page 449). The fat-free residue was extracted with 25 per cent alcohol, concentrated by vacuum distillation, then spread on dextrin and dried. Two animals fed Diet 149 plus doses of this extract equivalent to 1 gram per day of lichens, which was increased on the 35th day to 2 grams, gradually lost weight and both died on the 47th day. The food consumption was 102 and 127 grams respectively for the entire period, which was lower than for the control animals on Diet 149 used in the present experiment. It appeared evident that this lichen extract was distinctly unpalatable like the whole lichen.

Vitamin A

The vitamin A estimations were made by the therapeutic method. Young rats were placed in individual cages at the age of 21 or 22 days and fed a vitamin A-deficient diet (No. 158) which consisted of: alcohol extracted casein, 18 per cent; dextrinized rice starch, 72 per cent; agar, 1 per cent; irradiated dry yeast, 5 per cent; mineral mixture, (Osborne and Mendel) 4 per cent. When further gains in weight ceased on Diet 158, the diets containing lichens were substituted. This change in diet was made, in the usual case, between the thirty-seventh and fortieth days. Various levels, ranging from 2 to 20 per cent of the short and from 2 to 10 per cent of the tall lichens were substituted in Diet 158 in place of proportionate quantities of dextrin.

The results on 38 rats are shown in Table II. The animals are grouped in the table according to percentage of lichens in the therapeutic diet. In a number of cases, animals fed the same level of the first and the second consignments of lichens are grouped together. The gains in weight during the therapy period are given in the table at the end of four, six, and eight weeks. In those cases where the animals died after four or six weeks on test feeding, the gains or losses in weight and the average daily feed consumption are given for the periods which the animal survived. The condition of the animals at the time of death or at the close of the experiment is also given.

The short lichens fed at a 2 per cent level relieved the avitaminosis condition in part and enabled three of the five rats to survive the therapeutic period. Two per cent of tall lichens were without benefit to three animals. All animals on the 5 per cent level of short lichens survived the experiments, showed only minor symptoms of vitamin A deprivation, and gained an average of 56 grams on a calculated daily consumption of approximately 0.5 grams of short lichens. This dosage was more than sufficient to

furnish one vitamin A unit (4) based on an average gain of 3 grams per week.

One, of a group of six animals, survived the eight-week test period on the 5 per cent level of tall lichens. The survival period was longer than on the 2 per cent level, which may be taken to indicate that the tall lichens contain at least small amounts of vitamin A. However, all five animals on a 10 per cent level of tall lichens died within four weeks, due primarily to a lack of vitamin A.

A 200-gram sample of tall lichens was dried at reduced pressure in an atmosphere of carbon dioxide for two hours at 70°C., then extracted with absolute alcohol. The alcoholic extract was removed from the flask at frequent intervals during the early part of the extraction in order to avoid long continued heating. At the end of six hours, the alcohol was entirely drained off the lichens and ether added. The extraction was continued for eight additional hours. The combined extracts were concentrated under reduced pressure and finally taken up in a hydrogenated vegetable fat free of vitamin A. This concentrate was fed to three rats to test its vitamin A potency. One rat received the equivalent of 0.5 gram and two others of 1.0 gram of lichens daily which corresponded roughly to the amounts on the 5 and 10 per cent levels respectively. This absolute alcohol-ether extract did not cause a pronounced decrease in food consumption such as was observed on an equivalent amount of unextracted tall lichens. The lichen extract also failed to arrest the development of characteristic symptoms of avitaminosis-A. It appears evident that the tall lichens contained considerably less vitamin A than the short lichens.

Vitamin D

The estimation of the antirachitic properties of lichens was carried out by the therapeutic method. Young rats were fed for four weeks on the Steenbock-Black rachitogenic ration which consists of yellow corn meal, 76 per cent; wheat gluten, 20 per cent; calcium carbonate, 3 per cent; and sodium chloride, 1 per cent (5).

At the beginning of the fifth week, the therapeutic diet was substituted. The lichens of Series 2 replaced equal amounts of corn in the rachitogenic diet at levels of 2, 5, 10, and 20 per cent. The eight days on therapy were followed by an additional day on the rachitogenic diet to allow for further assimilation and action of the antirachitic principle. At the end of this period the animals were weighed, killed, and the rear leg bones removed for ash analysis and line tests. The tibiae were placed in 10 per cent

TABLE II

THE RESULTS OF FEEDING TESTS WITH RATS ON THE VITAMIN A CONTENT OF LICHENS

Lichens	Sex and initial weight	Gain depletion	Condition and gain on therapy			Daily feed consumption	
Type, series and per cent			4 weeks	6 weeks	8 weeks	Depletion	Therapy
	grams	grams	grams	grams	grams		
Basal diet 158 without supplement	37♂	144	Died 19 days (++++)*			12.6	
	40♂	137	−89 died 28 days (++++)			11.6	
	31♀	72	Died 19 days (++++)			8.3	
	31♂	52	" 19 " (+++)			6.4	
	35♀	35	3	−17	−36 (++++)	10.6	7.5
Short (1) 2–10	45♂	152	−40	−17	−16 (+)	12.5	11.5
" (2) 2	33♂	73	16	23	Died 47 day (++++)	6.8	
	27♀	53	17	4	" 42 " (++++)	6.6	
	35♀	70	20	−2	14 (+)	8.7	7.7
	34♀	66	20	25	26 (+)	7.7	8.1
Tall (1) 2	36♂	113	Died 27 days (+++)			9.8	
	44♀	75	" 19 " (+++)			9.4	
	42♂	106	" 19 " (+++)			10.9	
Short (1) 5	38♂	112	49	63	69 (−)	11.9	12.7
" (2)	33♂	62	39	72	59 (+)	7.4	9.6
	40♂	26	60	86	69 (+)	5.1	10.2
	33♀	67	30	41	42 (+)	10.4	9.3
	29♀	18	29	45	43 (+)	7.4	7.2

Tall (1) 5	45♂	166	−60	−76	−91 (++)	13.4	10.2
	32♀	99	Died 28 days (+++)			9.8	
" (2)	35♂	70	−25	Died 40 day (++++)		8.4	5.9
	36♂	99	−36	" 42 " (++++)		9.7	
	30♀	64	−34	" 33 " (++++)		9.8	
	30♀	49	−19	" 37 " (++++)		6.9	
Short (2) 10	39♂	60	61	87	94 (−)	10.8	12.2
	33♂	76	51	91	111 (−)	6.8	13.2
	34♀	58	51	73	81 (−)	8.1	7.2
Tall (2) 10	33♂	62	Died 8 days (+++)			9.1	
	31♂	81	" 26 " (++++)			8.9	
	28♂	63	" 12 " (++++)			6.7	
	29♀	63	" 19 " (+++)			9.5	
	30♀	38	" 9 " (+++)			6.2	
Diet 158 plus alcohol 0.5 grams‡ ether-extract tall lichens 1.0 gm.	37♂	134	−59	Died 40 days (++++)		10.1	
	36♂	131	−76	Died 31 days (++++)		10.4	
	35♂	59	Died 20 day (+++)			6.1	

* The symbols − and + represent gradings of the condition of the animals relative to stages of vitamin A deprivation from a grade of − for nearly normal, then + to +++ for advancing stages of vitamin A deprivation.

‡ The quantities given are equivalents of lichens as the daily dose, that is, such quantity of extract was added as the daily dose as was obtained from 0.5 to 1.0 grams of lichens.

TABLE III

ANTIRACHITIC VALUE OF SHORT AND TALL TYPE LICHENS AS DETERMINED BY FEEDING EXPERIMENTS WITH RATS

Type and per cent of lichens in curative diet	Depletion period 28 days		Curative period—8 days					
	Total gain	Daily food	Total gain	Daily food	Daily doses lichens	Line test	Bone analysis	
							Ash	A/R
	grams	grams	grams	grams	grams		per cent	ratio
Negative controls	19	7.9				—	36.9	.59
	15	7.0				—	39.8	.69
	20	8.7				—	35.1	.54
Short type lichens	19	5.4	6	7.5	.38	—	38.7	.63
5 per cent	11	6.6	5	7.0	.35	—	32.0	.47
	24	8.4	2	7.0	.35	+	38.6	.63
	15	8.3	2	2.1	.11	++	42.6	.73
10 per cent	20	6.9	−6	8.0	.80	++	44.8	.81
	20	7.6	−2	5.1	.51	+	35.4	.55
	19	6.0	3	9.0	.90	++	43.1	.76
	13	9.0	1	9.6	.96	+	43.6	.77
20 per cent	10	6.1	−2	6.8	1.35	++	45.6	.84
	18	6.5	−3	8.3	1.65	++	36.6	.58
	4	7.7	5	10.1	2.02	++	43.1	.76
Tall type lichens	11	7.8	−7	6.9	.14	++++	44.9	.81
2 per cent	4	7.7	−5	6.8	.13	+++	48.5	.94
	9	7.7	−4	6.8	.13	++	46.9	.88
5 per cent	19	7.6	0	6.0	.30	++++	44.3	.81
	16	8.1	3	6.6	.33	+++	37.1	.59
	30	7.4	4	7.4	.37	+++	38.3	.62
	18	7.9	11	6.2	.31	+++	48.2	.93
ad. lib.	21	6.9	−3	3.8	.28	—	38.5	.63
10 per cent	15	8.0	−10	6.4	.64	++++	43.5	.76
	23	8.4	−6	4.8	.47	++++	40.0	.67
	19	7.5	−8	7.6	.76	+++	47.7	.91
20 per cent	18	6.3	−18	3.5	.70	++++	43.4	.77
	22	7.1	−10	5.0	1.00	++++	45.5	.84

formalin and line tests made according to the technic outlined by Bills, Honeywell, and McNair (6). Photomicrographs of the "line tests" have

greatly facilitated interpretation of the readings (7). The femurs were carefully cleaned of adhering flesh, dried, weighed, crushed with a haemostat, placed in a paper capsule, and extracted in a Soxhlet apparatus with alcohol and then ether for 12 to 14 hours each. The residue was dried, weighed, and ashed. The percentage of ash based on the extracted bone weight and the A/R ratio (ash to organic residue) as proposed by Chick, Korenchevsky, and Roscoe (8) were calculated.

The results are given in Table III. The animals used as negative controls, which were killed at the end of the four-week depletion period, all showed uniformly negative line tests. Of the groups on the three levels of short lichens, the one on 10 per cent showed definite healing in all cases.

On the 2 and the 5 per cent levels of tall lichens, definite healing was fully evident as shown by the line test readings. Above these levels there is evidence in certain cases of abnormal healing, presumably due to partial starvation superimposed upon the healing induced by the lichens. Analyses on the tall lichens showed 0.13 per cent and 0.03 per cent respectively of calcium and phosphorus. Accordingly, no additional calcium was required in the diets to maintain the 5:1 ration of calcium to phosphorus.

The results indicate that the short lichens induced healing of rachitic rats at a 10 per cent level, while the tall lichens were somewhat more effective at a level of only 2 per cent. In this connection it is of interest to note that Zellner (9) has reported the isolation of erogsterol in a lichen, *Peltigera canina*.

SUMMARY

Feeding tests with rats failed to show the presence of the factors constituting the vitamin B-complex in either short or tall type lichens at the levels at which it was possible to feed them.

Tests for the vitamin A response showed that 5 per cent of short lichens added to the basal diet during a curative period permitted a moderate rate of gain. No vitamin A could be demonstrated in the tall lichens, either when fed whole or as an alcohol-ether extract at levels up to or equivalent to 10 per cent of the diet.

The tall lichens apparently possessed greater antirachitic properties than the short lichens. Curative tests showed a marked difference at a 5 per cent level.

The tall lichens were not palatable to rats and few survived for any extended time when fed at levels greater than 10 per cent. The short lichens were well tolerated at the levels fed.

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EFFECT OF CRANBERRIES ON URINARY ACIDITY AND BLOOD ALKALI RESERVE

By

C. R. FELLERS, B. C. REDMON, AND E. M. PARROTT

(From Massachusetts State College, Amherst)

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THE effect of eating cranberries on human blood alkali reserve has not been reported in the world's scientific literature. Aside from the single experiment reported by Blatherwick and Long (1) in 1923, there are likewise no data bearing on the effect of cranberries on urinary acidity. These investigators found both prunes and cranberries when ingested in large quantities (about 300 grams per day) produced marked increases in both the titratable and organic acid acidities of the urine. The prunes contained 0.149 per cent benzoic acid and produced about double the acidity of a similar weight of cranberries with a benzoic acid content of .096 per cent. These amounts of benzoic acid being far too small to account for the large amounts of hippuric acid found in the urine, Blatherwick and Long conclude that the principal source of hippuric acid in the urine is probably quinic acid. Lücke (10) observed that the excretion of hippuric acid was elevated after the ingestion of many vegetables and fruits, especially cranberries. Quick (12) agrees with Blatherwick and Long that quinic acid is the substance present in fruits which is responsible for the increased excretion of hippuric acid. Kohman and Sanborn (9) have recently isolated quinic acid from both cranberries and prunes. There is no direct, accurate method of determination. Quick (12) demonstrated that quinic acid in the human organism was first transformed to benzoic and finally to hippuric acid. The transformation takes place more slowly than with benzoic acid and elimination is incomplete even after 24 hours. In general, elimination of 80 per cent or more of the benzoic acid occurs within six hours after ingestion.

In the conjugation of benzoic acid with glycine to form hippuric acid, there is no evidence of a deficiency of glycine to bring about this change unless abnormally high doses of benzoic acid are ingested. The glycine utilized for the conjugation normally is not derived entirely from the diet but is synthesized as needed. It is presumed though not proved that glycine is synthesized largely by the liver. Swanson (13) concludes that glycine is probably synthesized from the constituents which are normally converted

to urea. Griffiths and Lewis (7) demonstrated that the rate of excretion of hippuric acid was decidedly increased by the administration of glycine alone with the benzoic acid.

Other substances which have been shown to form hippuric acid in the body are benzaldehyde, cinnamic acid, and toluene. The first two of these substances occur normally as glucosides in various natural food products but are not known to occur in cranberries.

OBJECT OF RESEARCH

The object of the present research was to determine in human subjects the effect of ingested cranberries on the composition of the urine and on the blood alkali reserve.

METHODS

Six male human subjects, from 22 to 27 years of age, were used in these experiments. They are designated as P, R, N, J, C, and W and were given a daily basal diet consisting of:

Graham crackers	300 grams
Milk (3 pints)	730 grams
Iceberg lettuce	120 grams
Heavy cream (½ pint)	225 grams
Eggs (2)	140 grams
Apple (1)	150 grams

In the second experiment (Table II) subjects N and J followed this diet with the exception that they ate no apples. In the third experiment (Table III) the basal diet was followed with the exception that Subject C used no cream and ate two apples a day. Subject J ate two apples a day in place of one. The basal diet was eaten for several days or until the body reached a metabolic equilibrium, after which cranberries were eaten in the desired amounts.

Three experiments were conducted. The first afforded a means of observing the effect of the ingestion of large amounts of cranberries and was made on Subjects P and R (Table I). The second was run in an effort to determine the rate with which the body recovered from the effects produced by eating large quantities of cranberries. Subjects N and J were used in this experiment the results of which are tabulated in Table II. The third experiment was made on Subjects C, W, P and R using small normal servings of cranberries (Table III).

The Kingsbury and Swanson (8) hippuric acid technic was used and found very satisfactory. Urea nitrogen was determined by the urease

TABLE I

EFFECT OF LARGE DOSES OF CRANBERRIES ON URINARY ACIDITY AND BLOOD ALKALI RESERVE

Date July, 1931	Subject	Volume cc. per 24 hrs.	Sp. gr.	pH	Grams in 24 hours								Blood* alkali reserve cc.	Diet
					Nitrogen		Creatinine	Phosphorus	Hippuric acid	Uric acid	Tit. acid	Org. acid		
					Urea	Ammonia								
5 day av. 14–18	R	871	1.022	6.26	10.58	0.45	1.01	1.30	0.80	0.33	397	446	76.7	Basal
	P	1599	1.012	5.96	10.70	0.74	1.30	1.08	0.78	0.31	474	517	71.7	
19	R	710	1.023	6.1	10.44	0.20	0.73	1.20	1.57	0.18	465	558	61.7	Basal+100 grams
	P	1200	1.015	5.8	12.36	0.26	0.93	1.00	1.64	0.28	478	566	57.3	cranberries
20	R	880	1.021	6.1	9.56	0.44	0.92	1.33	2.49	0.29	456	608	—	Basal+150 grams
	P	1170	1.015	5.7	9.46	0.54	1.04	0.96	2.46	0.33	451	665	—	cranberries
21	R	775	1.020	6.2	8.32	0.46	0.80	0.93	3.00	0.24	363	600	42.0	Basal+200 grams
	P	1945	1.012	5.8	10.56	0.94	1.30	0.89	3.88	0.31	451	702	40.2	cranberries
22	R	1000	1.018	6.1	8.66	0.44	1.05	1.16	3.16	0.32	442	614	—	Basal+250 grams
	P	1065	1.018	5.6	8.36	0.52	1.11	0.91	3.40	0.39	443	682	—	cranberries
23	R	1080	1.015	5.8	7.24	0.58	0.87	1.09	4.35	0.22	492	694	29.6	Basal+300 grams
	P	1840	1.010	6.2	7.94	1.06	1.23	0.74	4.48	0.17	398	764	25.8	cranberries

* CO_2 per 100 cc. plasma.

TABLE II

EFFECT OF CRANBERRIES ON URINARY AND BLOOD ACIDITY. RATE OF RECOVERY

Date Aug. 1931	Subject	Volume	Sp. Gr.	pH	Grams in 24 hours					Alkali** reserve plasma cc.	Remarks
					Nitrogen		Hipp. acid	Tit. acid	Org. acid		
					Amm.	Urea					
Av. 3–4	N	1260	1.015	6.6	0.54	10.03	0.82	282	526	81.4	Basal diet
	J	610	1.030	5.75	0.44	9.83	0.93	469	585	62.5	
5	N	1190	1.015	6.3	0.60	11.00	5.52	354	649	51.0	(after 2 hrs.) Basal diet plus
	J	430	1.030	6.2	0.48	8.72	0.90*	292	419	49.1	254 grams cranberries
6	N	1015	1.018	6.5	0.52	8.88	1.75	360	732	34.3	(12 hrs.) Basal diet
	J	580	1.030	5.8	0.52	8.78	3.15	460	647	44.2	
7	N	1635	1.012	6.6	0.64	9.14	1.45	307	557	77.3	(36 hrs.) Basal diet
	J	540	1.033	5.9	0.43	9.15	0.74	462	530	72.6	
8	N	1000	1.018	6.6	0.50	8.60	0.93	282	546	73.6	(60 hrs.) Basal diet
	J	605	1.028	6.2	0.55	9.16	0.87	363	590	67.6	

* On this date Subject J failed to collect the early morning sample of urine which undoubtedly contained practically all the hippuric acid for that day as the cranberries were eaten about 6 o'clock the evening before.

** CO_2 per 100 cc. plasma.

method as described by Folin and Youngburg (6), uric acid by the Christman-Ravwitch modification of the Folin and Franke method (2), and organic acids by the Van Slyke and Palmer technic (15). Ammonia N was determined by the Folin and Bell method (5); creatinine by Folin's method (4) and phosphorus by the Fiske and Subbarow method (3).

The figures in the tables are expressed in terms of grams per 24 hours in all cases except titratable acidity and organic acids which are reported as cubic centimeters of one-tenth normal acid in 24 hours. The alkali reserve is expressed as cubic centimeters of carbon dioxide in 100 cubic centimeters of blood plasma. In the last two experiments it was deemed unnecessary to determine creatinine, phosphorus, or total nitrogen, since these values showed no particular variation.

Blood samples were taken at varying hours following the consumption of cranberries. The experimental day began and ended at 8 a.m. Cranberries were eaten at noon and evening in the first experiment, at 6 p.m. in the second and at noon in the third experiment. Urine samples were kept in the refrigerator to prevent bacterial decomposition.

Both Howes and Early Black cranberries were used, usually in the form of whole or strained cranberry sauce. However, all results are calculated to weight of fresh fruit.

DISCUSSION

Table I contains detailed data on the various constituents of urine as well as on blood alkali reserve.

In general the data on volume, specific gravity, pH, creatinine, and phosphorus are of little significance and show no important changes during the course of the experiments. Both titratable and organic acids are increased following consumption of large quantities of cranberries. Doses of 100 grams or more of cranberries in a 24 hour period increased the titratable and organic acids in the urine not over 15 per cent in any case.

Hippuric acid gave the best gauge of the quantity of cranberries ingested, the quantity found in the urine being directly proportional to the weight of cranberries eaten. The only exception is found in Table III where small dosages of cranberries (22 to 54 grams) daily showed but slight increase in hippuric acid.

Since upon analysis the cranberries showed from 0.05 to 0.1 per cent benzoic acid, the balance of the hippuric acidity must have originated in some other substance viz., quinic acid. Thus indirectly, we find that cranberries contain from 0.5–0.8 per cent of quinic acid. Since quinic acid acts in the same manner as benzoic, in forming hippuric acid in the human

body, we can thus account for the increased acidity of the urine following the eating of cranberries.

Even small amounts of cranberries, Table II, 22 to 54 grams, equivalent to from ? to 5 ounces of cranberry sauce, produced slight increases in urinary acidity, though from a physiological or health viewpoint this slightly increased acidity is without significance. These amounts probably represent average servings of cranberries. Table II shows that the recovery of urinary acidity to normal after eating large doses of cranberries may be delayed for 48 hours though a large part of the hippuric acid is voided during the first 24 hours after eating the cranberries. This experiment confirms Quick's (12) results which show benzoic acid is eliminated from the body within a very few hours after ingestion while quinic acid is eliminated much more slowly.

Since in the determination of organic acids, hippuric acid is also included, the same variations occur in this determination as were just discussed for hippuric acid. The hydrogen ion concentration of the urine was usually slightly increased by eating cranberries, the increase being approximately 0.2 for large doses of cranberries. Uric acid appears to decrease slightly in the urine. Quick (12) explains this decrease in uric acid excretion on the basis that all the glycine present in the kidney may be withdrawn to detoxify the benzoic acid when large amounts are fed.

The ammonia content of urine increased a little after eating cranberries. The urea was perceptibly decreased, the nitrogen which normally is eliminated as urea may be required to form the glycine which in turn is necessary for the conjugation and elimination of benzoic acid as hippuric acid.

Cranberries are thus seen to increase both titratable and organic acidities, hippuric acid, and ammonia, while urea nitrogen, uric acid, and pH are decreased.

Blood Alkali Reserve

Alkali reserve of the blood was determined by means of the Van Slyke apparatus (14). The carbon dioxide gas, from the bicarbonates, is liberated from a measured sample of blood plasma and its volume carefully determined. Duplicate and sometimes triplicate determinations were made. Individuals vary a great deal in their blood alkali reserve and a value that is normal for one person may be quite abnormal for another. However, by using the same individual in feeding tests involving alkali reserve, comparative and conclusive results may be obtained.

The data in Tables I and II are largely self-explanatory. Large doses of cranberries have a marked effect in decreasing the blood alkali reserve.

TABLE III
EFFECT OF SMALL AMOUNTS OF CRANBERRIES ON URINARY ACIDITY AND BLOOD ALKALI RESERVE

TABLE III

EFFECT OF SMALL AMOUNTS OF CRANBERRIES ON URINARY ACIDITY AND BLOOD ALKALI RESERVE

Date Aug., 1931	Subject	Vol. urine 24 hrs.	Sp. gr.	pH	Grams in 24 hours					Blood* alk. res. (Plasma) cc.	Diet	
					Nitrogen		Hippuric acid	Titratable acid	Org. acids			
					Urea	Amm.						
Av. 16–18	C	795	1.021	6.2	9.87	.413	1.12	351	527	75.5		Basal
	W	1218	1.016	6.6	8.88	.577	1.35	291	544	77.6		Basal
19	C	870	1.018	6.4	9.58	.52	1.24	332	—	73.6	(19 hrs.)	Basal+22 grams of
	W	1660	1.012	6.7	10.25	.72	1.45	267	673	73.9	(5 hrs.)	cranberries
20	C	960	1.020	6.6	9.96	.46	1.21	280	—	74.2		Basal+22 grams of
	W	1215	1.021	6.2	8.70	.60	1.53	408	607	78.8		cranberries
21	C	1115	1.018	6.6	9.45	.55	1.33	276	475	79.5	(12 hrs.)	Basal+22 grams of
	W	920	1.020	6.3	9.17	.49	1.20	363	—	79.5	(2 hrs.)	cranberries
June, 1932						Uric acid						
Av. 14–15	R	893	1.024	6.65	10.4	.46	1.01	269	516	71.7		Basal
	P	1420	1.015	6.2	9.8	.43	0.90	366	572	69.3		Basal
16	R	940	1.018	6.0	10.7	.49	1.07	425	504	67.4	(4 hrs.)	Basal+32.7 grams of
	P	1535	1.015	6.2	10.9	.44	1.29	435	589	75.2	"	cranberries
17	P	1250	1.025	6.2	11.2	.42	1.63	400	500	73.6	"	Basal+32.7 grams of cranberries
18	P	1680	1.015	6.5	9.9	.48	1.20	320	408	75.5	"	Basal+54 grams of cranberries
19	P	1630	1.020	6.1	10.4	.41	1.77	350	462	73.1	"	Basal+54 grams of cranberries
20	P	1480	1.020	6.2	10.5	.41	0.84	348	420	73.1	"	Basal

* CO_2 per 100 cc. plasma.

Quantities of cranberries varying from 100 to 254 grams (equivalent to 9–24 oz. of cranberry sauce) reduced the blood alkali reserve from 30 to 60 per cent. This degree of acidosis may be considered mild to moderate.

As shown in Table III, smaller amounts of cranberries, 22 to 54 grams, corresponding to 2–5 oz. of sauce, produced no demonstrable acidosis. It thus appears that the blood buffer substances are able to maintain the alkali reserve and *p*H even in the presence of moderate amounts of free acids. Though cranberries in normal servings do not lower the blood alkali reserve, they apparently do not leave an alkaline residue in the body in spite of the slight alkalinity of the ash (11).

SUMMARY

When cranberries were fed in amounts of 100–300 grams daily to normal young men the following observations were made as to the composition of the urine:

1. The titratable acidity, organic acids, hippuric acid, hydrogen ion concentration, and ammonia were increased, while uric acid and urea nitrogen were slightly decreased.

2. The amount of hippuric acid recovered in the urine was roughly proportional to the weight of cranberries eaten.

3. The hippuric acid was largely voided over a 24 hour period following the ingestion of cranberries.

4. The origin of most of the hippuric acid in the urine is presumably quinic acid or a glucoside which produces quinic acid upon hydrolysis. The cranberries used contained only 0.05 to 0.09 per cent benzoic acid, an amount totally inadequate to account for the large amounts of hippuric acid recovered in the urine.

5. By measurement of the hippuric acid content of the urine and the benzoic acid content of the cranberries ingested, the conclusion is reached indirectly that cranberries contain from 0.5 to 0.9 per cent quinic acid in free or combined form.

6. Two to five ounces of cranberry sauce equivalent to from 22 to 54 grams of fresh cranberries produced only very slight increases in urinary acidity. These quantities of cranberry sauce probably represent average servings.

7. The decrease in urea nitrogen in the urine is probably due to the additional quantities of glycine required for the conjugation of the benzoic acid in the body. The glycine may make use of nitrogen which would otherwise be voided in the urine as urea.

8. Large quantities of cranberries decrease the carbon dioxide combining capacity of the blood, the decrease being in general proportional to the quantity of berries eaten. The usual decrease after eating 100–300 grams of cranberries was 30 to 60 per cent and produced a mild to moderate acidosis.

9. When normal quantities of cranberries were eaten, no decrease in blood alkali reserve was found. Thus two to five ounces of cranberry sauce, equivalent to 22 to 54 grams of fresh cranberries, produced no demonstrable decrease in blood alkali reserve.

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THE STIMULATING ACTION OF COPPER ON ERYTHROPOIESIS*

By

HERMANN B. STEIN AND ROBERT C. LEWIS

(From the Department of Biochemistry, University of Colorado School of Medicine, Denver)

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ALTHOUGH numerous investigations on the effect of copper in the presence of iron on the cure and prevention of nutritional anemia have been made during recent years, few workers have studied the effect of copper *without iron* on animals receiving a basal diet of milk. Among these Titus and Hughes (7), Waddell, Steenbock, and Hart (9), and Keil and Nelson (1) have followed only the hemoglobin in such studies, while Schultze (4) has made simultaneous erythrocyte counts and hemoglobin determinations. Before the latter paper appeared, the investigation herein reported on the effect of copper without iron on both hemoglobin and erythrocytes had been undertaken in our laboratory (5) incidental to certain investigations with evaporated milk.

EXPERIMENTAL

Albino rats were taken from their mothers when 21 days old, placed in all-glass cages, and kept on a diet of evaporated or raw milk, the latter supplemented with copper in some cases, as indicated under experimental results.

The cage was essentially that described by Underhill, Orten, and Lewis (8), although some modifications of design have been made. Short glass rods long enough only to separate the tubing which constitutes the floor were employed in place of the glass-tube side walls of the original cage. For the side walls, double strength window glass measuring 9 1/4 × 27 1/2 inches was used. These were held together with strips of adhesive tape measuring 1×18 inches. The same size glass was used for the top of the cage, the wooden frame being omitted. To prevent movement, the top was held in place by **U**-shaped glass tubing wide enough to span the width of the cage. The solid glass sides served to hold down the glass tubes used as flooring, thus eliminating the long glass tubes employed for this purpose in

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the original design. This arrangement also permitted the glass partition to rest directly upon the wooden frame, thereby making unnecessary the use of an extra glass tube alongside the partition to prevent contact of rats in neighboring compartments. As seen in the illustration (Fig. 1), the partitions are held in place by long glass rodding uprights. This cage can be taken apart and cleaned very quickly.

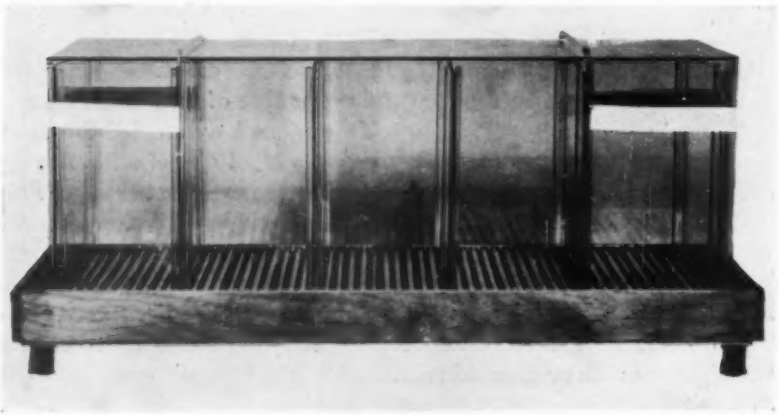

FIG. 1.—Modified glass cage.

The raw milk used was collected directly from the cow into wide-mouthed jars, a worker from the department supervising each collection. The evaporated milk, that purchasable on the open market, was made up as required from the large (14 1/2 ounce) can by dilution with an equal amount of water distilled from glass, and was kept in wide-mouthed glass jars. Both the raw milk and the diluted evaporated milk were kept in the refrigerator. The halves of standard Petri dishes used for feeding were washed in soap and water, placed in approximately 10 per cent nitric acid for 24 hours, then rinsed in water distilled from glass, and finally allowed to drain and to dry spontaneously. The milk was transferred to the feeding dishes by one-ounce "Asepto"[1] syringes, which were easily used and cleaned.

The copper was fed as copper sulfate in an aqueous solution of such strength that 0.5 cc. contained 0.25 mg. of copper, or, if less copper was

[1] Becton, Dickinson, and Company, Rutherford, New Jersey.

used, 0.025 mg. It was prepared from C.P. copper sulfate recrystallized several times from redistilled water.

The animals were weighed weekly, and hemoglobin determinations and erythrocyte counts were made biweekly at first, then weekly. The blood was obtained from the young rat by clipping the tail with a safety razor blade held in a patented holder. The tail was cauterized with a long, narrow strip of window glass, one end of which had been heated in a gas flame. As the animals became larger, it was possible to obtain blood by nicking one of the vessels of the tail. If bleeding did not stop quickly, a rubber band was used as a tourniquet by wrapping it around the tail a couple of times and holding it in place with an artery clamp.

For blood counts the regular Thoma diluting pipette was used, and Hayem's solution served as the diluent. The count was made in a Levy-Hauser counting chamber. The earlier hemoglobin determinations were made with the Hellige double glass standard Sahli apparatus. Simultaneous determinations made with the Bausch and Lomb Newcomer hemoglobinometer checked the Sahli readings within narrow limits. Later, a technic for the Newcomer apparatus providing greater speed and convenience with no loss in accuracy was used as follows: Five cc. of N/10 HCl were run from a burette into each of the required number of test tubes. The tubes were then closed with rubber stoppers and placed in a metal test tube rack. Each blood was collected in the regular Newcomer pipette and expelled into one of the test tubes, the pipettes being rinsed a few times by drawing up the acid and expelling it. The tube was shaken to mix the contents, and then replaced in the rack. The used pipette was washed in water and alcohol, and attached to a vacuum, where it dried in a few minutes and was ready for use again. The test tubes were allowed to stand for an hour before being read from the same source of artificial light, with the instrument placed the same distance from the light each time. This technic was convenient and accurate, and permitted the use of a minimum number of pipettes.

RESULTS

The rats (5 animals) which were fed raw milk alone showed a sharp fall in hemoglobin and red cell count. In contrast the hemoglobin of the rats (5 animals) given diluted evaporated milk fell less rapidly, while the erythrocytes increased. These results, shown graphically in Chart 1, indicate that the evaporated milk contains an erythropoietic agent not found in raw cow's milk.

Since the milk comes in close contact with copper during the manufacturing process employed in the majority of evaporated milk plants, it seemed possible that copper absorbed by the milk might be the responsible agent for the erythropoiesis. Supplee and Bellis (6) in 1922 found that, when milk was passed through tin-lined copper pipes from which the tin was worn, the copper content increased as much as 2.0 mg. per liter.

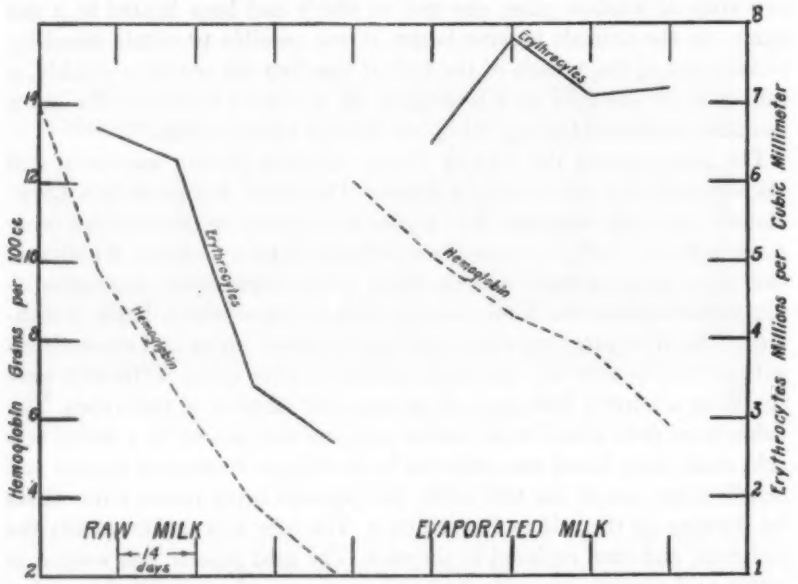

CHART 1.—Composite curves (average of 5 animals) showing the comparative effect on the hemoglobin and the erythrocyte count of feeding exclusive diets of raw milk and evaporated milk, respectively, to rats.

Rice and Miscall (3) reported that the amount of copper taken up by milk increased with the amount of copper surface to which the milk was exposed and with the length of exposure. Since these reports thus indicated that there would be a significant increase of copper in processed milk, an investigation was undertaken to determine if this metal was the causative factor in the maintenance of the red cell count when evaporated milk was fed. Consequently, 0.025 mg. of copper, the amount employed most often in nutritonal anemia studies, was fed daily to each of a group of normal rats (6 animals) receiving raw milk as the basal diet. The results are shown in Chart 2. The hemoglobin fell gradually, but the number of erythrocytes

at the end of two weeks remained unchanged. During the next two weeks there was a slight increase in the red cell count followed by a gradual decrease in successive periods. The relatively slow fall in the erythrocyte count, as compared with that obtained when raw milk without copper is fed, indicates that copper plays a rôle in erythropoiesis.

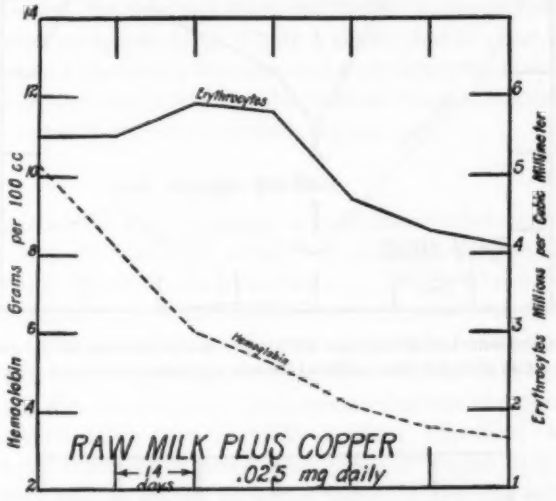

CHART 2.—Composite hemoglobin and erythrocyte curves (average of 6 rats) showing the effect of copper when added as the sole supplement to raw milk in maintaining the erythrocyte count at a relatively high level.

To confirm this supposition, an experiment was planned to see if copper would have an actual stimulating effect by increasing the red cell count of anemic rats. For subjects of these experiments, rats (4 animals) were fed raw milk alone until they became markedly anemic. After six weeks, when the red cells averaged 3,482,000 per cu. mm. and the hemoglobin, 3.4 gm. per 100 cc., 0.25 mg. of copper (ten times the previous dose) was given daily as a supplement to the milk diet. Chart 3 shows that during the next five weeks the erythrocytes increased to 5,725,000 per cu. mm. while the hemoglobin only rose to 4.75 gm. per 100 cc. Thus, it is evident that copper has an erythropoietic action independent of any influence it may have in the presence of iron on hemoglobin regeneration.

In the work of Schultze, to which reference has been made, larger amounts of copper (2 mg. daily), when given to anemic rats, produced an increase in the red cell count with little, if any, effect on the hemoglobin.

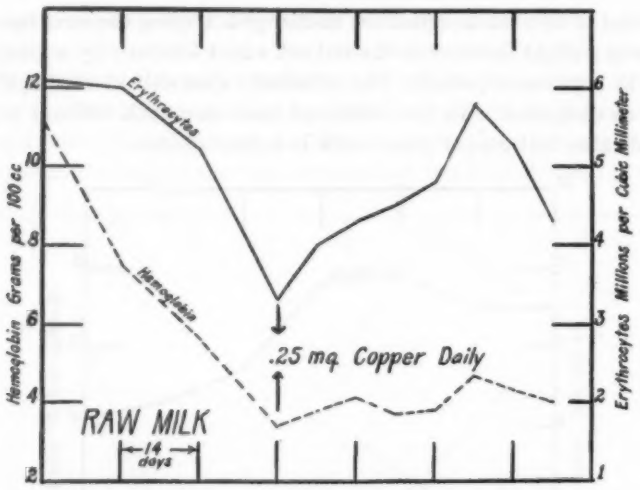

CHART 3.—Composite hemoglobin and erythrocyte curves (average of 4 rats) showing the erythropoietic action of copper when added as the sole supplement to the raw milk diet of anemic rats.

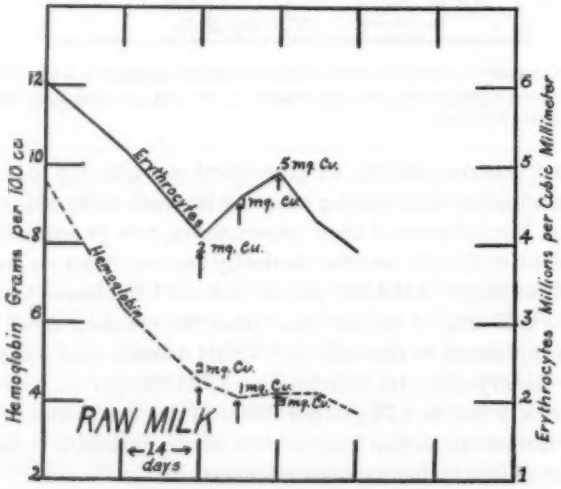

CHART 4.—Composite hemoglobin and erythrocyte curves (average of 4 rats) showing a relatively slight erythropoietic action when large doses of copper are added as the sole supplement to the raw milk diet of anemic animals.

When an attempt was made to duplicate these results by giving this larger dose of copper (2 mg.), it is interesting to note that the animals developed a severe diarrhea with an accompanying loss of weight. As it was evident that copper in this amount was toxic, the amount of this metal given daily was reduced after one week to 1 mg. daily. Since the diarrhea and loss of weight continued, the dose was decreased further at the end of the second week to 0.5 mg. of copper daily. Chart 4 shows that in these rats (4 animals) there was a temporary increase in the erythrocyte count with no rise in hemoglobin, thus confirming the observations made in the earlier experiments that copper has an erythropoietic action.

DISCUSSION

These observations that copper has a stimulating effect on erythropoiesis distinct from its "catalytic action" on hemoglobin formation do not receive as much support in the literature as would have been the case had hemoglobin determinations and erythrocyte counts both been made in the studies on nutritional anemia which have appeared in recent years. In such experimental work, where iron and copper have both been given as supplements to a milk diet, the results of the independent effect of copper on erythrocyte formation have been partially masked. However, Myers and Beard (2) have shown that, when small doses of copper (0.0025 to 0.01 mg.) are given daily to anemic rats on a milk-iron diet, the red cell count returns to normal in most instances at a considerably more rapid rate than does the hemoglobin. This would seem to indicate, though not definitely, that copper has a stimulating effect on erythropoiesis independent of its effect on hemoglobin formation.

More clear-cut results have been obtained by Schultze (4) in experiments where copper *without iron* has been added as a supplement to the milk diet of anemic rats. Under these conditions the lack of iron prevents hemoglobin formation, inasmuch as this element is a necessary constituent of the hemoglobin molecule. Thus, the possibility of confusing the effect of copper on erythropoiesis with its catalytic action on hemoglobin formation is avoided. Schultze has found, as we have done, that a rise in the erythrocyte count with no change in the hemoglobin follows the administration of the copper.

Another point of considerable interest revealed by the experimental data may be observed by the examination of Charts 1 and 2. It will be seen that the rate of fall of the hemoglobin with a diet of raw milk alone is much more rapid than when either evaporated milk or raw milk supple-

mented by copper has been fed. Evidently the presence of copper, both in the evaporated milk as well as when used as a supplement to raw milk, exerts its well known catalytic action on hemoglobin formation, even in the absence of a dietary source of iron, by bringing about a better utilization of tissue iron or of the iron liberated during the process of destruction of hemoglobin in bile formation.

On the basis of our experimental findings, supported by the work of others, we feel justified in drawing the conclusion that copper has two distinct rôles in blood formation: first, its well recognized catalytic action on hemoglobin formation, a point on which all workers in the field of nutritional anemia agree, and second, a stimulating effect on erythropoiesis.

SUMMARY

1. The feeding of evaporated milk to normal rats produces a fall in hemoglobin without the corresponding drop in the erythrocyte count that occurs when raw milk is the sole article of diet.

2. When copper without iron is fed as a supplement to raw milk, results similar to those with evaporated milk are obtained, indicating that the copper in the evaporated milk is responsible for the temporary maintenance of a high erythrocyte count.

3. When given as a supplement to the milk diet of anemic rats, copper ranging in amounts from 0.025 mg. to 0.5 mg. daily shows a definite erythropoietic action without any influence on hemoglobin formation.

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Editorial Review

THE POSSIBILITY OF THE CONVERSION OF FATTY ACIDS TO GLUCOSE IN THE ANIMAL BODY

OF ALL the possible interconversions of food nutrients in the animal body, the conversion of fats to sugar seems to have excited the most intense interest and to have divided scientific opinion the most sharply. The reasons for this unique characterization are not far to seek.

The conversion of protein to sugar in the diabetic animal and the conversion of sugar to fat in the heavily-fed farm animal are, and have been, readily demonstrated. The rapidity of conversion is so great as to be unmistakable. On the other hand, the conversion of sugar or fat to protein is out of the question, because the latter nutrient contains nitrogen and generally sulfur, two elements not found in sugar or fat. The fact that sugar and some of its probable metabolites, in conjunction with ammonium salts or some other simple nitrogenous compound, may be synthesized to one or two of the simpler amino acids is interesting, but falls far short of establishing any reasonable probability that from such precursors a complete protein molecule can be formed in the animal organism. The conversion of protein to fat in the higher animals is not easily proven, it is true, and at the present time, in the reviewer's opinion, it has not been rigorously demonstrated. Nevertheless, the evidence in favor of the conversion is weighty, and the similarities in the structure of some of the amino acids and of fatty acids, together with what is known of their metabolism, offer no serious obstacles to the belief that the conversion is possible. Furthermore, the transformation of protein to fat through sugar, though indirect and cumbersome, is thoroughly possible. Doubtless some day an ingenious experiment will be devised that is capable of establishing definitely that protein is convertible into fat, but there is little incentive (and unfortunately not much credit to be gained) merely to demonstrate what is almost universally believed to be true.

Contrast with these problems that of the conversion of fat to sugar, neglecting the glycerol constituent of the former. For almost fifty years it has engaged the interest of scientists, and yet so slow is the rate of conversion, if it exists at all, or so restricted the conditions permitting it, or so difficult is it to devise an effective experimental method, that the mass of

evidence is utterly confusing. As reviewed by some investigators, it is interpreted to mean that the conversion has been proven; other reviewers are as positive that the conversion is impossible, while the more moderate verdict of "not proven" is voiced by others. There are no *a priori* grounds for believing the conversion to be possible in the body. The difficulties inherent in the problem and the divergent views concerning its solution have both served to sustain interest in it, and today it is being as vigorously attacked as at any time since it was first conceived.

It will not be the purpose of this review to consider all of the experimental work concerned with the possible conversion of fatty acids to sugar. The many earlier reviews of this question, particularly the excellent critical discussion of Rapport (32) published in July, 1930, make such a course unnecessary. A general consideration of the different lines of approach will be attempted, but only those contributions appearing within the last two or three years, and in a few cases earlier studies intimately related to them, will be appraised in any detail. It appears unnecessary to discuss the glycerol constituent of fats in this connection, since the conversion of this compound to sugar in the diabetic organism, as well as in the phlorhizinized dog, has been firmly established.

The problem of the convertibility of fatty acids into sugar is related to other important physiological problems so intimately that its solution follows almost as a corollary from the solution of the latter. An example of such a related problem is that of the nature of the metabolic disturbance in diabetes. If this disturbance is essentially a partial or complete failure in the ability of the tissues to oxidize glucose, there are no implications concerning the ability of the body to form glucose from fatty acids. On the other hand, if the metabolic disorder is solely an over-production of glucose, the mechanism of glucose oxidation being unimpaired, then the low respiratory quotients in diabetes can be explained only on the assumption of a massive conversion of fat into sugar. The latter theory of diabetes has never been securely established, and in the last few years it has become less tenable than ever (9, 34, 35). In this country, at least, it is commonly believed that the "defective oxidation theory" of diabetes explains the observed facts much better than the "over-production theory."

Another illustration of the same point is the problem of the fuel of muscular contraction. Among a number of influential groups of physiologists in England, Germany, and Canada the theory that the muscles, and probably other tissues, can burn only sugar has been strongly advocated. Obviously if this theory is correct, fatty acids must be convertible into

sugar, because fats are demonstrable sources of muscular energy. MacLeod, a member of one of these groups, has called sugar the "fuel of life" and has written a book in defense of this thesis. The most direct evidence bearing upon the problem and responsible for this particular solution of it has been obtained, first, from chemical and thermodynamic studies of isolated muscle in various conditions of rest, activity, fatigue, and recovery, and, second, from observations on the respiratory exchange of intact animals during and following muscular activity.

The classical investigations of muscle chemistry and muscle energetics initiated in 1911 by Fletcher and Hopkins and continued brilliantly by Hill, Meyerhof and their collaborators, established many facts concerning muscular metabolism, facts that revolutionized current conceptions and opened up many avenues for further investigation. But in interpreting these facts in terms of concrete chemical reactions and in applying them to the muscle *in situ*, it now appears that they arrived at a solution of the problem which was much too simple. They assumed that the only chemical reactions occurring in isolated muscle that contributed appreciably to the energy transformations or the gaseous exchanges were the reactions involved in (or conditioned by) the anaerobic breaking down of glycogen to lactic acid in an initial phase, and the oxidative reconstruction of glycogen from lactic acid in a recovery phase at the expense of lactic acid, or possibly some other metabolite of glucose with an R.Q. of 1. Thus, the only source of muscular energy, according to this simple picture, is glucose, and the conversion of fatty acid to glucose becomes a necessary corollary. The further assumption that no other source of muscular energy is available to the muscle *in situ* appeared to be substantiated by a number of experiments on men during and after short periods of moderate muscular exertion. In such experiments, regardless of the value of the basal respiratory quotient, the quotient of the excess gaseous exchange approximated closely to 1. Hill has described, correlated and interpreted these different lines of evidence in a very readable article that appeared in *Science* for December 5, 1924.

Since then many important contributions have been made to our knowledge of muscle chemistry and muscle physiology, and the carbohydrate changes have assumed a secondary role. It appears that the anaerobic production of lactic acid furnishes, not the energy for muscular contraction as was formerly believed, but the energy to reverse the reaction of first importance, the anaerobic dissociation of phosphocreatine (phosphagen), thus recharging the muscle (30, 24). In this newer physiology, less cer-

tainty is expressed in the nature of the material that furnishes the energy for the resynthesis of glycogen in an isolated muscle, but even though this ultimately may prove to be carbohydrate in nature, there is no good reason to believe that the muscle *in situ* is so restricted in its choice of fuel. A tissue removed from the body has never been known, in so far as the author is aware, to acquire a function it formerly did not possess, but it obviously may lose a function that was dependent upon an intact supply of blood, lymph, and nerve impulses or upon the coöperation of other organs and tissues of the body. In fact, much evidence has accumulated to the effect that the respiratory quotient of the excess gaseous exchange produced by contraction of the muscles in the intact organism may assume a wide range of values. While this does not disprove the theory that sugar is the immediate fuel of the muscles, to which all other nutrients including fat must be converted, it does detract from the necessity of incorporating it into any complete explanation of muscular metabolism. Perhaps one reason why this theory has persisted so long and has obtained such a wide currency is that it is impossible to disprove it by any known method.

It is a fair judgment, therefore, that these modern investigations of muscular metabolism, although they have been so interpreted, have established no considerable probability for the theory that fatty acids are convertible into sugar in the animal body. The well-established fact, often cited in this connection, that muscular activity is performed more economically when the source of energy is sugar than when it is fat is consistent with the theory that the latter must be converted to the former for complete utilization, but it does not amount to competent evidence for it.

If the conversion of fatty acids to sugar occurs in the body, its effect would be to depress the respiratory quotient, since it would amount to a storage of oxygen. If R.Q.'s less than that for the oxidation of fat, i.e., 0.70 or thereabout, could be secured experimentally under conditions of a plethora of fatty acid molecules in the blood and intercellular fluids, then objective evidence of the conversion would be at hand, quite analogous in its significance to R.Q.'s greater than 1.00, obtained after large carbohydrate meals. It is true, the evidence would not be so clear cut, because a conversion of amino acids to sugar, the desaturation of fatty acids, and various intermediary oxidative reactions may have the same effect, and it must also be remembered that the failure to obtain such low quotients after fat feeding is no evidence against the possibility of the conversion, because the effects of a slow rate of conversion on the respiratory quotient may be entirely obscured by the simultaneous oxidation of a small amount of protein or of sugar.

In Murlin and Lusk's (31) studies of the specific dynamic effect of fat they fed 75 grams of the nutrient to dogs. In many of the experiments no significant depression of the basal R.Q. occurred and in no case was the hourly quotient depressed below 0.71. Gregg (16) working in Murlin's laboratory has determined the respiratory quotients of albino rats (weighing 150 to 200 grams) after the consumption of relatively large amounts of butterfat. Of all of the quotients obtained, only one, 0.634, may be regarded as possibly indicating a formation of sugar from fatty acids. Ten other quotients were less than 0.70, but they occurred during the early hours of fat absorption and according to Gregg, "are due without doubt to a transient acidosis and ketonuria." The average of all respiratory quotients obtained is 0.72, and the entire series can be explained without the necessity of assuming a formation of sugar from fatty acids. In this connection, it should be emphasized that an occasional atypical R.Q. considerably below 0.70 cannot be accorded full significance, because this quotient is ordinarily subject to a technical error of ± 0.02 and occasionally a much greater error may be committed. Observations of similar import on men consuming meals containing as high as 200 to 300 grams of fat have been reported by McClellan, Spencer, and Falk (28).

These results are typical of high-fat diets, and offer no support for the theory that fatty acids are convertible to sugar in the body. However, some observations from Murlin's laboratory indicate the possibility of forcing the R.Q. below 0.70 by feeding high-fat diets. Hawley, Johnson, and Murlin (21) reported such observations upon human subjects and Hawley and Murlin (22) similar observations upon a pig. Unfortunately these experiments have not as yet been reported in detail. It appears that the low non-protein R.Q.'s were corrected for any products of incomplete fat metabolism present in blood and urine. In the pig experiments it is stated that low quotients were obtained following the meal with a subsequent rise above the original level. This sequence of events suggests that gastric secretion with its withdrawal of chloride from the blood, and intestinal absorption with its restoration of chloride, may have produced these variations in R.Q. by causing a retention, followed by an expulsion of carbon dioxide from the body. This question has been discussed by McClellan, Spencer, and Falk (28). Murlin himself, in a private communication, frankly admits that "of course the evidence from low quotients must be checked by actual finding of the carbohydrate which is supposed to be formed."

Inexplicably, to the reviewer at least, indications of the conversion of

fatty acid to glucose based upon the respiratory quotient, have been obtained more frequently in the post-absorptive period than in the period following excessive fat feeding. During the post-absorptive period the metabolic activities are presumably almost entirely catabolic in character, while the glycogen stores, if they are not at their maximum for the dietary regime prevailing, have not been greatly depleted. When, under such conditions, respiratory quotients less than 0.70 are obtained, they are ordinarily dismissed as the result either of technical errors in gas analysis, or of a fasting ketosis, representing incomplete oxidation. Thus, the low quotients reported by Heinbecker (23) for Eskimos may be considered unreliable, because of the low oxygen percentages obtained for atmospheric air, indicative of an ineffective pyrogallol solution.

Recently, Lyon, Dunlop, and Stewart (27) have reported low total respiratory quotients for obese subjects in basal metabolism tests. These subjects were on reducing diets, containing 2000 calories or less per day. The quotients below 0.70 were particularly frequent for the subjects on a diet of 1000 calories, containing only small amounts of carbohydrates. For 26 determinations, calculations of the non-protein R. Q., the maximal sugar that could have been produced from protein, and the minimum amounts of sugar that must have been produced from fat are made. Throughout all tests, a routine examination of the urine for acetone was made. In a number of instances when acetone was present, the total excretion of ketones was determined quantitatively, but was never found to exceed 0.5 gram daily. The Douglas bag method was used, and the collection periods were of six minutes duration. Every point of possible error in technic seems to have been checked and the authors feel justified in concluding tentatively that "our experimental results afford evidence that in the undernourished obese subject, fat is converted into carbohydrate."

In an experiment in which the accuracy of the most significant results depends so intimately upon the accuracy of gas analyses, it is disturbing to find that the analyzers were tested daily, not with outdoor air of known and constant composition, but with laboratory air, which ordinarily cannot be so characterized. The calculations of the non-protein R.Q. and of the extent of the possible conversion of protein to sugar and of fat to sugar are carried out according to approved methods. While the point is not seriously involved in the validity of the main contention of Lyon, Dunlop, and Stewart, it may not be entirely impertinent to comment on the uncertain reliability of such methods of analyzing the data obtained from the respiratory metabolism and the excretion of urinary nitrogen. All such methods

are based upon a simplified and purely conventional picture of metabolism, in which there is no place for transformations of one nutrient into another within the range of respiratory quotients from 0.7 to 1. They involve a correction for protein metabolism based upon the urinary nitrogen as a criterion of the rate of protein oxidation, and the factors used for estimating the heat liberated and the gaseous exchange resulting from protein oxidation relate to one protein, generally, in this country at least, the protein of meat. The lag in the excretion of nitrogen liberated by deamination, as well as the fact that deamination of amino acids may occur without their complete oxidation, do not deter investigators from applying these methods to all conditions of metabolism and rarely elicit any misgivings anent the significance of the results obtained. The meat protein factors are used in interpreting the course of metabolism after food, whether meat, or some other protein differing widely from it in nitrogen content and in digestibility, is being consumed, or whether the subject is a dog, the animal on which the factors were obtained, or a steer. They are used in the analysis of basal metabolism data, during a period when a considerable part of the urinary nitrogen is probably a delayed excretion from the catabolism of the last meal, and when the endogenous end products, representing apparently a type of tissue catabolism not involving simply a protein oxidation, account for an increasing fraction of the urinary nitrogen. Fortunately the untenable assumptions upon which this method of indirect calorimetry is based may not appreciably impair the accuracy of the estimations of total heat produced within the range of respiratory quotients from 0.7 to 1, if these estimations have been adequately checked by direct calorimetric methods.[1] This circumstance is due both to the fact that the heat equivalent of a liter of oxygen is not greatly affected by the nature of the metabolic mixture, and to the fact that the heat equivalent of a liter of oxygen consumed in the oxidation of protein is not greatly different from that of oxygen consumed in the oxidation of a mixture of fat

[1] However, Adams and Poulton (*Proc. Physiol. Soc.*, 1932, 1P; *Jour. Physiol.* 77) have recently presented some disturbing evidence, from published calorimetric data, that the error in indirect calorimetry relating to total basal heat production is correlated with the respiratory quotient. They interpret this correlation to mean that, regardless of the observed R.Q., a constant proportion of carbohydrate and fat is being burned corresponding to an R.Q. of 0.785. The writer has made a study, similar to that of Adams and Poulton, of selected data from Lusk's numerous calorimetric experiments on dogs, the basis of selection being (1) that the directly determined heat production did not follow the administration of cold liquids, and (2) that the calculated heat production did not involve any but the ordinary assumptions. From a compilation of about 300 data, not however confined to basal periods, no appreciable correlation was detected between the percentage difference between observed and calculated heat and the total respiratory quotient.

and sugar corresponding to an R.Q. of 0.80, characteristic of protein oxidation. Hence, large errors in estimations of the extent of the participation of sugar, fat, and protein in the energy metabolism may coexist with inconsiderable errors in the estimation of total heat production.

The contribution of Lyon, Dunlop, and Stewart is certainly suggestive of a conversion of fat to sugar, but the results of these experiments practically stand alone as regards the large proportion of basal respiratory quotients below 0.70. In the well-known studies of Means (29), some low quotients were obtained in individual tests, but they were not typical of any subject but one. Hagedorn, Holten, and Johansen (19), in basal metabolism studies on 16 normal and 30 obese subjects, obtained very few respiratory quotients less than 0.70, but they did note a distinct tendency for the quotients of the obese subjects to be lower than those of the normals, the difference averaging 0.05; all subjects were on the same high-carbohydrate diet. This difference is interpreted as the result of an abnormally increased transformation of carbohydrate to fat in the obese immediately after food, with a correspondingly lower respiratory quotient in the post-absorptive period.

The ability of the animal body to transform sugars, glycerol, lactic acid, and some of the amino acids into glycogen has been investigated by feeding these substances to experimental animals and then determining the concentration of glycogen in their livers after allowing a few hours for absorption. If the glycogen concentration is considerably higher in animals so fed than in control animals that received no food, the conclusion has been drawn that the substance fed has been converted to glycogen. While the evidence is not unassailable, since the participation of glycogen precursors from other tissues in the increase in liver glycogen cannot be ruled out, it may amount to practical certainty if the increases above the control level always occur and are always large. On feeding fats in such an experiment, no evidence has been obtained that fatty acids are convertible into glycogen. If animals are maintained for some time on a high-fat diet some glycogen formation is indicated, but no more than could be amply accounted for by the glycerol contained in the fats (16, 18). In a recent experiment, Greisheimer (18) fasted rats for 48 hours and then fed them different food materials during a 24 hour period; the amounts consumed were unfortunately not reported. The rats were then killed and the livers analyzed for glycogen. Four female rats fed lard showed an average concentration of 0.202 per cent of glycogen in the liver, as compared with the average of 9 control females of 0.135 per cent. Eight male rats gave an average of 0.715 per cent

glycogen against the control average (3 males) of 0.513 per cent. One male rat was forced fed with oleic acid, the amount given being again unstated. The liver glycogen in this case was 0.207 per cent. Contrasted with these negative results are the positive results obtained with glycerol, casein, sucrose, and the stock diet, which induced glycogen concentrations ranging from 1.81 to 7.82 per cent.

Experiments have also been carried out on the possible role of fatty acid metabolites in glycogen formation. Thus, Rosenthal (36) tested β-hydroxybutyric acid on fasting mice, but was unable to secure evidence of its conversion to glycogen in the liver. Eckstein (13) in similar experiments on fasting rats, investigated a series of lower fatty acids, including propionic, butyric, valeric, and caproic acids. Only propionic acid, an acid that has never been related to fat metabolism, gave evidence of conversion to glycogen in the liver. Butyric acid, a probable metabolite of naturally-occurring fatty acids, gave entirely negative results.

Another method of approaching the problem of the conversion of fats to sugar in the liver has been the perfusion experiment. Conflicting results have been reported in the literature, a situation not surprising in view of the fact that perfusion technic is extremely difficult and its refinement to anything approaching perfection is a very late accomplishment. At the present time it is realized that proper aeration of the excised liver by means of the perfusion fluid is extremely important, not only to the continued survival of the organ, but to the demonstration of its functional capacities. It is also evident that conclusions cannot be based merely upon the changes that occur in the composition of the perfusate. Particularly with reference to the problem under discussion, an examination of the changes that have occurred during the perfusion in the composition of the liver tissue itself is essential to a successful experiment.

In 1926, Burn and Marks (5) reported the results of a series of very carefully performed perfusion experiments on the livers of dogs and cats. In all experiments, increases in the total carbohydrate content of the perfusion system were observed, which could not be accounted for by decreases in lactic acid, or by increases in amino acid deamination and conversion of the non-nitrogenous residue into sugar. Although no attempt was made to determine whether the fat of the liver decreased during the experiments, the authors can conceive of no other source from which the sugar could have come.

The experiments of Burn and Marks have been critized by Richardson (33, p. 97) because of the probable formation of lactic acid in the livers

under the anaerobic conditions that prevailed during the 20 to 30 minutes required for preparation for perfusion. He assumes that this lactic acid may have been the source of the sugar produced. However, the rate of disappearance of lactic acid was computed from initial analyses of samples of the perfusate taken 5 minutes after perfusion started. Presumably the lactic acid previously formed in the liver was present in this initial sample, so that its rate of disappearance was fully evaluated. It may be that the data of Burn and Marks are subject to considerable technical error,[2] and that their attempts to rule out non-fat precursors of the carbohydrate apparently produced were not successful, but taking the results as they have been interpreted, they constitute no evidence for the conversion of fatty acids to sugar. The gains in sugar reported varied from 0.3 to 1.5 grams, equivalent, in the two cases for which the necessary data are reported, to 0.3 and 0.6 per cent of the weight of the perfused liver, part of which may, according to the author's own data, have originated from lactic acid or amino acids. The residue may have been produced from glycerol liberated from the fats or phospholipids present in the liver tissue.

Jost (25) has more recently attempted to apply liver perfusion to the solution of the same problem. The perfusion experiments were carried out both with livers from dogs in a normal nutritive condition, and hence containing presumably a normal storage of glycogen, and with livers from fasting phlorhizinized dogs, which were rich in fat and poor in glycogen. The most striking results of these experiments were obtained after the addition of a phosphatid (either lecithin or cephalin) to the fluid being perfused through the fat-rich, but glycogen-poor, livers. On such addition, the reducing power of the perfusate was increased and the respiratory quotient of the liver determined from blood analyses was depressed. On the basis of these (and other) results it was concluded, first, that the catabolism of higher fatty acids takes place with the intermediate formation of phosphatids, and, second, that sugar may be formed in the liver from the fatty acids liberated from the phosphatids.

However, the significance of the gaseous exchange of such a preparation may well be questioned, because of the changes occurring in the CO_2 capacity of the perfusate and the incomplete oxidations of various descriptions that may be occurring, quite aside from the hypothetical conversion it is desired to demonstrate. The increase in reducing power of the perfusate,

[2] Perhaps one indication of technical error is their failure to find any appreciable amounts of glycogen in the livers of fat-fed animals. This is quite a unique finding, contrasting with the recent findings of Gregg (17) and of Kapeller-Adler and Rubinstein (26).

if this may be taken to represent sugar only, may have been the result of a glycogenolysis induced by the lecithin (or cephalin) added to the perfusion fluid. The fact that little glycogen was found in the livers of the phlorhizinized dogs, does not mean that more might not have been present, since the determination of small amounts of glycogen by the Pflüger method yields minimal results (14). The effect of lecithin upon the carbohydrate metabolism of the intact animal seems to be a stimulation of glycogenolysis according to Cruto (10, 11). Its specific effects upon the liver of normal and depancreatized animals is being studied by Best and his associates (2, 3). The effects on the content of the liver in fat, phospholipins, and glycogen are suggestive of a conversion of fat to sugar, but a final decision on this question awaits the results of further study.

Gregg, working in Murlin's laboratory, has contributed the latest report (17) on liver perfusion, using the liver of fat-fed cats and dogs. In seventeen such experiments he observed the following in the perfusion system: a decrease in total carbohydrate, a decrease in total lipids, a steady production of urea, ammonia, and sugar, a smaller production of lactic acid and nonfermentable reducing substances, a marked constancy in the fatty acids of the perfusate, and a decided irregularity in the distribution of carbohydrates in livers containing them in small concentrations. No support for the theory of gluconeogenesis from fatty acids was obtained, and Gregg expresses the following significant judgment of the value of the perfusion method in the solution of this problem:

"Due to the changes necessarily inherent in such an unphysiological system, namely, lactic acid formation, glycogenolysis, metabolic use of sugar by the liver, edema of the perfused organ associated with marked unevenness of the distribution of sugars, and finally, the ineradicable possibility that sugar may always be coming from glycerol, it is well nigh impossible to use such a system to demonstrate the neogenesis of carbohydrate from fatty acids on a quantitative basis."

The diabetic state has always been considered a fruitful one for the demonstration of the conversion of nutrients or metabolites into sugar. Furthermore, it may readily be induced experimentally by pancreatectomy. The poisoning of animals with phlorhizin also induces a condition similar to pancreatic diabetes in that gluconeogenesis is greatly stimulated, while sugar oxidation is correspondingly inhibited.

The post-absorptive respiratory quotient in either clinical or experimental diabetes is generally low, and not infrequently below the R.Q. for fat. This, however, is not indicative of the conversion of fatty acids to

sugar, since the metabolic derangements in diabetes modify profoundly the significance of the quotient, particularly the incomplete oxidations of fatty acids and amino acids and the accumulation of acidic metabolites in the body fluids. Although the calculations designed to gauge the effects of these derangements of metabolism upon the respiratory quotient are not particularly convincing, it does appear that their net effect will be to lower the oxidative respiratory quotient. Hence, it does not appear to be necessary in this case to enlist the services of a hypothetical conversion of fatty acid to sugar.

Phlorhizined dogs will readily produce sugar from proteins and many of the amino acids, and the formed sugar is excreted so promptly and so invariably that it has been possible to compute the yield of sugar from different proteins and even the number of carbon atoms in any glucogenetic amino acid that is carried over into sugar. But the ingestion of fat by phlorhizinized dogs has elicited no such response in the large majority of experiments of this type, and the result is the same when fats are fed to diabetics. In those few studies in which the production of extra sugar has been claimed, the amount is so small and the dextrose to nitrogen ratio in the urine so variable that the evidence is of doubtful validity. For example, in Geelmuyden's experiments (15) the effect of fat on the sugar excretion of phlorhizinized rabbits was tested with 5 animals. In three experiments the nitrogen in the urine as well as the urinary sugar was determined. In only two of these cases was any disturbance in the D:N ratio produced by fat feeding, and the slight disturbance noted occurred, not on the day of fat-feeding, but on the second and third day following. Obviously, in the two experiments in which the urinary nitrogen was not determined, any disturbances noted in the excretion of urinary sugar cannot be profitably discussed.

Probable metabolites of the higher fatty acids, such as butyric and acetic acids, are also without effect on the D:N ratio of phlorhizinized animals. Geelmuyden's experiments (15) are again quite negative in their significance, although interpreted in a positive fashion by the author, who consistently refused to give to an increased nitrogen excretion in such experiments its customary and logical significance. Deuel and Milhorat (12), in much better controlled experiments on phlorhizined dogs, observed no indications of a conversion of acetic acid to sugar.

Soskin (37) attempted to induce a conversion of fatty acids to sugar in depancreatized dogs by feeding them fat (either olive oil, cottonseed oil, butter, or "intarvin") with lipase, or lecithin. The test substances were

given to the dogs on the fifth day after the withdrawal of food and insulin. In 10 of 13 experiments, Soskin was unable to secure evidence of the conversion of fatty acids to sugar, but in 3 experiments, the same type of calculation indicated the excretion of amounts of sugar (5.28, 4.85, and 6.91 grams) that were not traced to glycerol or catabolized protein. In all of these cases the dogs died on the day following the experimental feeding, which consisted, in one case of 40 grams of lecithin (15 grams given subcutaneously), in the second case, of 50 grams of olive oil and 50 cc. of castor bean suspension, and in the third case, of 30 grams of lecithin. The 10 negative experiments, in all of which the dogs survived, merely illustrate in the opinion of Soskin "the physiological limitations of the method."

In the 3 experiments interpreted in a positive fashion the R.Q.'s were not so low as to suggest a conversion of fatty acid to sugar, being 0.69, 0.71, and 0.71, and the possibility that glycogen stores might have been the source of the extra sugar as calculated is dismissed as "unlikely. There is at least no evidence that the administration of a food material like a neutral fat can cause a displacement of glucose from the tissue stores and it can be seen from the tables that the control animals in the present experiments showed no such displacement." The control animals were given mineral oil. No critical comment on these experiments is called for. Their mere description indicates the intangible character of the evidence adduced.

Much controversial material has been published concerning the significance of the dextrose to nitrogen ratio in clinical and experimental diabetes. This controversy has revolved mainly around the contention that, in pancreatic diabetes, the ratio assumes a fairly constant value approximating 2.8 to 1, while in phlorhizin diabetes it approximates 3.6 to 1, and that these ratios measure the extent of the conversion of protein to sugar under the respective conditions. Since the discovery of insulin and its use in preserving indefinitely the lives of depancreatized animals, it has been possible to study the dextrose to nitrogen ratio under better controlled conditions and on dogs in a much better physical state. Variations have been observed in the ratio which have been interpreted as evidence that, after the glycogen stores are depleted, other nutrients than protein are contributing to the urinary sugar. Chaikoff (6) believes that there is a relation between the ratio and the condition of the animal such that fat animals tend to exhibit a high ratio and thin animals a low one. Arguing that D:N ratios greater than 3.65 constitute "indisputable evidence that sugar is derived from fatty acid," when proper allowance is made for the possible

formation of sugar from glycogen stores and glycerol, Chaikoff concludes that the data for one of his four dogs (Dog C) support the view "that glucose is derived from fatty acid in the diabetic organism."

The argument is illogical, since it employs a D:N ratio the significance of which rests upon the assumption that fatty acids do not contribute to sugar formation, to prove that fatty acids are in fact so convertible. Furthermore, in this and all other experiments of this type it is impossible to rule out all participation of glycogen stores in the formation of urinary sugar on the basis of estimates of the probable glycogen content of the animal from averages of analyses on other dogs. This is true, first, because of the variations exhibited among dogs with respect to their glycogen stores: a small group of analyses affords no sure basis for setting an upper limit to the glycogen stores compatible with a given condition. In particular, as Rapport (32) has aptly pointed out, fat (well-nourished) dogs, which showed the suspiciously high ratios, may be expected to contain exceptionally large stores of glycogen as well as of fat, all the more so if Wertheimer (38) is correct in his finding that adipose tissue itself may contain as much as 1 per cent of glycogen.

A second objection to such calculations rests on the inaccuracy of the glycogen determination itself (14), but particularly upon the glycogenolysis that invariably occurs previous to analysis of muscle and liver. Cori (8, p. 162, 174, 175) has clearly described and emphasized these sources of error, particularly the glycogenolysis following death: "When an animal is killed by stunning, bleeding, decapitation, administration of ether or chloroform, the resulting asphyxia as well as strong nervous discharges, both acting at a temperature of 37°C., cause a rapid breakdown of glycogen. A muscle which is removed under these conditions even if it be frozen at once shows a lactic acid content which is considerably above the normal resting value and also considerably higher than the lactic acid content of the blood." As an indication of the order of magnitude of the error thus induced, Cori cites a value of $0.37 \pm .03$ per cent of glycogen in the gastrocnemii of fasted rats after killing, and a value of $0.59 \pm .04$ per cent when the muscles were removed from the living animal under amytal anesthesia. Anderson and MacLeod (1) have more recently studied this post mortem glycogenolysis, particularly with reference to the death of the muscles themselves, and have stated that "no method was found in which the muscle could be killed without causing stimulation of the reaction leading to the disappearance of glycogen. A post mortem increase of lactic acid, usually out of proportion to any glycogen decrease, was always found under

these conditions." It must be concluded that all glycogen analyses of animal tissues possess a minimal significance, and that, under the conditions that usually obtained in published experiments, they may be very greatly in error.

In 1926 Wertheimer (39), convinced that fats are convertible to sugar, tested the possible catalyzing effect of insulin on the reaction. He compared the reaction to insulin, first, of dogs whose bodies had been depleted of glycogen in a preliminary phlorhizin period and whose livers had accumulated an abundance of fat, and, second, of dogs that had been subjected to fasting only or had had their normal ration up to the time of insulin administration. He found that the phlorhizinized dogs were less sensitive to insulin shock than the unphlorhizinized dogs, many of which died shortly after the insulin injection. Their blood sugar returned to normal levels quicker (or even attained hyperglucemic levels); their livers lost fat, but gained glycogen. Hence, the conclusion was drawn that fats had been converted to sugar, which exerted its well known antidotal effect on insulin shock. The possibility that protein was the precursor of any sugar or glycogen formed is dismissed with the statement that protein catabolism was unchanged, though an increase in protein catabolism is characteristic of effective phlorhizinization of dogs. Wertheimer's interpretation of his experiment ascribes to insulin a function that no other experiments concerned with the physiological effects of the hormone have even suggested, insofar as the reviewer is aware, and the experiment, viewed in the light of this interpretation, may almost be said to "prove too much" in implying that, in the insulinized animal, sugar is more readily produced from fat than from glycogen.

The conception of Wertheimer has been submitted to a much more thorough test by Hawley (20), who used the same dogs, first in a fasted and then in a phlorhizinized condition, for insulin treatment. She also determined the respiratory exchange of the dogs during the first 6 to 8 or 9 hours after administration of insulin. In these experiments, the animal after phlorhizinization was in no case more resistant to insulin than after fasting, and the respiratory quotients in no case suggest a conversion of fat to sugar. It is true that a negative experiment of this type cannot, by that fact alone, invalidate an experiment possessing an opposite significance, but its results may be given more weight if they are based upon a more comprehensive set of data involving less biological error, as these were, and if the interpretation that they support more readily dovetails in to the established facts and generally accepted theories of metabolism.

The primary effect of epinephrine on carbohydrate metabolism is an acceleration of glycogenolysis in all cells. When epinephrine is injected into a depancreatized dog an increase in the D:N ratio may follow, the extra glucose originating from the glycogen stores of the tissues. Repeated injections generally have failed of this effect. In 1928, Chaikoff and Weber (7) attempted to account quantitatively for the sugar excreted in the urine of depancreatized dogs in response to epinephrine injections. On the third and fourth days after the last meal and the last injection of insulin, depancreatized dogs were given repeated injections of epinephrine. The extra sugar excreted in the urine was compared with the sugar that might have been formed from liver glycogen, protein, or glycerol, and in 8 of 9 tests, the sugar formed exceeded the given sources of sugar. Although the authors believed that muscle glycogen could not, on the basis of published work, have contributed to the urinary sugar, its possible effect was nevertheless estimated. With this added source of sugar, there were still three tests in which the extra urinary sugar exceeded the sum of the quantities of sugar from all recognized sources. Although the sugar and glycogen contents of the animals were not determined either before or after the experiments, being estimated on the basis of previous analyses on dogs in similar condition, Chaikoff and Weber concluded that the extra sugar, in some of the experiments, must have been derived in part from fatty acids.

Rapport (32) has criticized the experiments of Chaikoff and Weber on the following points: the glycogen stores were not stabilized at the time of epinephrine injection, since the D:N ratios had not reached a level; the impossibility of making any accurate estimate of the "extra sugar" excreted because of the extreme variability of the dextrose to nitrogen ratios in the urine; the assumption that muscle glycogen could not have contributed to the extra sugar; the respiratory quotients are not in harmony with the assumed conversion of fat to sugar. To these criticisms may be added the probability of considerable error in the assumed glycogen contents of both muscle and liver. The question of the possibility of muscle glycogen contributing to epinephrine glycosuria was answered very definitely in the affirmative by Bollman, Mann, and Wilhelmj (4), who repeated the experiments of Chaikoff and Weber, but analyzed samples of muscle, blood and liver of each dog, four being used, both before and after the period of epinephrine injection, throughout which time the urine was obtained by catheterization. Their calculations show, not only that the muscle glycogen stores are depleted by epinephrine, but that the sugar thus formed amply accounts for the sugar in the urine in excess of that

produced from protein; the glucose and glycogen of the liver and the glucose of the muscle were not appreciably changed. It may be noted that though all glycogen analyses made on muscle or liver may be much lower than the true values, for reasons already given, the differences in the values obtained before and after the epinephrine injections may have been substantially correct, if the glycogenolysis initiated by the removal of tissue samples may be assumed to proceed to a like equilibrium determined by the concentration of lactic acid capable of inhibiting further change. These experiments of Bollman, Mann, and Wilhelmj may be considered to dispose completely of Chaikoff and Weber's interpretation of their own experimental data.

This review of recent contributions to the problem of the possible conversion of fatty acids to sugar demonstrates the elusiveness of the definite solution. The methods of experimental attack are indirect, perhaps necessarily so, and the logic involved in the interpretation of the results so often involves debatable propositions, or such naïve expectations as that in fat animals the catabolism of fat and hence the rate of its conversion to sugar, are more intense than in thin animals, or that a glycogenolytic agent such as epinephrine should catalyze reactions leading to the formation of more sugar, or possibly more glycogen, to feed the glycogenolysis mechanism. Without exception the evidence for the conversion is not directly associated with fat, but, by a process of more or less successful elimination, with a substance that cannot conceivably (to the investigator himself) be anything else but fat. Granting the validity of the evidence, such an association may be tantamount to a demonstration, but on the other hand it may be merely a result of an incomplete state of knowledge of animal metabolism, or even a revelation of a mental bias, or of a lack of imagination in the investigator. The significance of most of the information yielded by these indirect attacks on the problem must be discounted, if it is not entirely invalidated, by later investigations that reveal unsuspected technical errors, or furnish alternative explanations of the data, or prove that the data are, for some unexplained reason, quite atypical of the experimental conditions.

The sum total of the evidence for the possibility of a conversion of fatty acids to sugar seems to be merely suggestive, certainly far from conclusive. On the other hand, there is no justification for a catagoric denial that the conversion is possible. The verdict of "not proven" is the most logical one to return.

In concluding this discussion it may not be presumptuous to suggest a

new line of attack. The mammary gland is concerned with the continuous secretion of sugar, and possibly with its continuous manufacture. The milk formed by the gland is passed to the exterior where it may be weighed and analyzed. If this secretion can be maintained on a high-fat diet in any species of mammal, without destruction of body protein and after depletion of body glycogen, at such a rate that the non-fat constituents of the diet could not support it, a high probability could be established that dietary fat was the source of the lactose formed. Negative results would of course possess little significance, since fat may be a specific inhibitor of lactation.

H. H. M.

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